Ethnogeology at the Core of Basic and Applied Research:

Surface Water Systems and Mode of Action of a Natural Antibacterial Clay

of the Colombian Amazon

by

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ABSTRACT

Amazonia, inhabited and investigated for millennia, continues to astonish scientists with its cultural and natural diversity. Although Amazonia is rapidly changing, its vast and varied landscape still contains a complex natural pharmacopeia. The Amazonian tribes have accrued valuable environmental and geological knowledge that can be studied. This dissertation demonstrates that Indigenous Knowledge considered alongside Western Science can enhance our understanding of the relationship of people to geological materials and hydrological resources, and reveal mineral medicines with practical applications.

I used methods from anthropology and geology to explore the geological knowledge of the Uitoto, a tribe of the Colombian Amazon. The Uitoto use two metaphors to describe Earth systems: 1. the earth is a body, and 2. the Amazon is a tree. I found that they classify surface-water systems according to observable characteristics and use mineral clays to treat various maladies. I argue that Uitoto knowledge about Amazonian mineral resources and surface water is practical, empirically–based and, in many cases, more nuanced than mainstream scientific knowledge.

I studied the mode of action of a natural antibacterial clay from the Colombian Amazon (AMZ) to discover whether the Uitoto's claims about the clay's medicinal values was verifiable using the methods of Western Science. Natural antibacterial clays can inhibit the growth of human pathogens. Methods from microbiology and geochemistry were combined to evaluate the mineral-microbe interactions that inhibit

growth of model Gram-negative (*Escherichia coli*) and Gram-positive (*Bacillus subtilis*) bacteria. The AMZ antibacterial clay contains 45 % kaolinites and 30 % smectites. Its high surface area maintains an acidic environment (pH 4.5) and releases high concentrations of aluminum. Aluminum accumulates in the outer membrane of *E. coli* by binding to phospholipids. Furthermore, the membrane's permeability increases due to synergistic effects between aluminum and transition metals released from the AMZ (i.e. Fe, Cu). The changes in the membrane may compromise its function as a barrier. Understanding the antibacterial mechanism of AMZ is key for its safe use as a natural product. These findings can help us harness the capabilities of antibacterial clays more efficiently.

Lastly, I integrated the results of this work in place-based, cross-cultural educational materials tailored for the tribal schools in the Colombian Amazon. The design of the units was informed by principles of curriculum design and successful pedagogic approaches for Native American students. The purpose of these educational materials is to return the results of research, enhance learning and participation of indigenous peoples in geosciences, and respond to the multicultural and plurilingual educational needs in countries such as Colombia.

Dedicated to

Tonantzin Tlali

Aiño komeki iji nóggora po

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PREFACE

Science (from the Latin *scientia*, knowledge) is in a very broad sense any systematic attempt to produce knowledge about the world through observation and evidence (Harding, 1988). Science is embedded in a culture, and is a culture in itself (Ogawa, 1986; Harding, 1988; Aikenhead, 2001). Scientists define which questions are worth pursuing (and often, what research merits funding), determine what constitutes acceptable evidence, and what is valuable knowledge.

Indigenous peoples from Amazonia such as the Uitoto, my collaborators in this study, have accumulated empirical knowledge by direct interaction with the natural world, and that knowledge has helped them thrive in the tropical rain forest. This body of environmental knowledge has been called Native science or Traditional Ecological Knowledge (TEK; Berkes, 1993; Cajete, 2000). The scientific study of cultural geological knowledge and the relationships between peoples and earth systems (i.e., earth materials, structures, processes, resources, hazards, and history) is called *Ethnogeology* (Kamen-Kaye, 1975; Semken, 2005). Ethnogeology has developed fairly recently compared to other ethnosciences but, like ethnobotany and ethnoecology, it has great potential to increase our understanding of some of the lesser-known aspects of tropical environments. Ethnogeology can also contribute to ethnopharmacology by helping us identify minerals with potential uses in medicine.

Traditional Ecological Knowledge, and ethnogeology, are unique to a group of people and specific to local environments and ecosystems (Eijck & Roth, 2007). I asked the questions: Can ethnogeology and Western Science increase our knowledge about the

mineral resources found in the Amazon? And, can we apply Western Science to further advance and universalize that knowledge? To explore these questions, I used ethnogeology in both basic and applied research.

Structure and Outline of the Dissertation

This dissertation is divided into three parts. In Part I, which includes chapters 1 and 2, I explore the geological knowledge of the Uitoto with respect to surface water systems and healing clays. This part is guided by two questions: (1) What is the geological knowledge that the Uitoto people have about their land? (2) How does that knowledge compare to Western science? In Part II, which includes chapters 3 and 4, I investigate a natural antibacterial clay from the Colombian Amazon and its mode of action. In Part III, chapter 5, I present place-based, multicultural instructional materials designed for indigenous students in the Colombian Amazon. The lesson plans for grades 4 and 5 focus on Earth systems and integrate local knowledge and surroundings.

With this dissertation, I demonstrate that Western science and indigenous knowledge can be combined to enrich our geological understanding of key resources like water, to foster scientific discoveries in medical geology, and to contribute cross-cultural educational materials to Indigenous communities

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CHAPTER 1

EXPLORING THE GEOLOGY OF THE COLOMBIAN AMAZON WITH INDIGENOUS EYES: ETHNOGEOLOGY OF THE UITOTO TERRITORY

Abstract

Colombia is both a biologically and geologically diverse country. The Colombian Amazon is home to half of the ethnic groups that live in Colombia. This rich setting offers a unique opportunity to study its geology using an ethnoscientific approach, drawing from both indigenous and Western science. This paper reports geological knowledge of the Uitoto people, a tribe living in the Northwest Amazon basin. Methods from anthropology and geology were combined to conduct an ethnogeological study of the Uitoto Territory, including its geology and salient resources. I use healing clays as an example for specialized indigenous knowledge that can correlate to Western Science. Study findings provide material that can be used to design cross-cultural lessons for the Natural Sciences for indigenous schools in Colombia. Our methods and findings could also be leveraged to teach ethnogeology–oriented courses at the college level. Such uses would advance educational practices in Geoscience that respect cultural diversity, include native knowledge, and fit the cross-cultural needs of students in countries like Colombia.

Introduction

As individuals and societies we adapt our lives to the geological processes that encompass us; we name and give meaning to the landscapes that surround us; and we use and appraise the rocks and minerals that underlie us. The scientific study of cultural geological knowledge and the relationships between peoples and Earth systems (i.e., Earth materials, structures, processes, resources, hazards, and history) is called *ethnogeology* (Kamen-Kaye, 1975; Semken, 2005). Ethnogeology can be the bridge between the allegedly disconnected Traditional Ecological Knowledge and Western Science, identifying similarities and differences.

The indigenous systems of knowledge can correspond to Western (or mainstream) Science (e.g., Snively & Corsiglia, 2001). Geologic knowledge accumulated by indigenous peoples can share the purposes and motivations of mainstream geology, e.g., cross-generational transfer of awareness of potential geologic hazards (Barber & Barber, 2004), locating subsurface metal ores (Tang, 1984), gaining knowledge about cycles and climate (Kronik &Verner, 2010), and managing limited resources such as water (Snead, 2006). Transmission of environmental information may have been key for survival.

Ethnogeologic studies have informed the development, distribution, and assessment of place-based education in geosciences for Native American and other indigenous peoples (e.g., Semken, 2005, Ward et al., 2014). Place-based education leverages students' senses of place by drawing on surrounding environments and landscapes, including local and indigenous ways of knowing and addressing relevant local issues and concerns (Semken & Butler Freeman, 2008). Further, a number of recent

studies (compiled by Apple et al., 2014) have shown that place-based teaching fosters interest and participation in geosciences studies and careers by underrepresented students from indigenous and historically resident groups.

To develop cross-cultural, place-based curricula that uses ethnogeological knowledge, educators need to have access to cultural knowledge, identify correspondences and contrasts between Native and Western bodies of knowledge, and understand the cultural framework from which each arises. This chapter is a step in that direction. The geological knowledge of the Uitoto about their land was investigated and intersections between indigenous and Western geological knowledge were identified. This information could be used to inform locally situated place-based education.

The Uitoto

The Uitoto (also Huitoto, Witoto, Murui-Muina, or Muinane) are a Native tribe from the northwest Amazon. Their traditional homeland is located within the interfluves of two main Amazon tributaries: The Caquetá and Putumayo rivers. Traditionally they hunt, fish, practice slash and burn agriculture in gardens or *chagras*, and gather wild products (Pineda–Camacho, 1985). They speak Uitoto, part of the Uitotoan linguistic family (Seifart, 2007), a language that has four related dialects. The Uitoto of Araracuara, the main focus of this chapter, speak Uitoto *N̲ɨ̲pode*[1] and Spanish.

[1] In the orthography developed by Griffiths et al., 2000, there is frequent use of the vowel barred i [ɨ], a high central vowel, pronounced similarly to English 'just' for some speakers. Accent marks show the place of the phonetic stress. In this paper we use italics and underline for words in Uitoto and italics for words in a language other than English.

The Amazon Basin

The Amazon drainage basin is one of the largest in the world. It occupies an area of about 8 million km^2 and contains the largest forests on Earth (Sioli, 1984). The upper basin is shared by the countries of Colombia, Perú, Ecuador, and Bolivia. The Colombian Amazon basin is limited to the west by the eastern Cordillera of the Andes, and to the north by the Vaupés swell, which divides the Amazon and Orinoco basins (Mora et al., 2010).

Figure 1.1. Regional map of the study area. AR: Araracuara Range, ChR: Chiribiquete Range. MR: Macarena Range. Modified from Google earth, 2015.

The Vaupés swell is a basement promontory. It forms the low Chiribiquete, Macarena, and Araracuara ranges (Figure 1.1) that expose the Guiana shield and

Paleozoic rock units at elevations up to 1,000 meters above sea level (m-asl). Thus, the geology of the Colombian Amazon is distinct from the middle and lower Amazon basin in that the sedimentary cover is thin and the compositions of the sediments are influenced by both the Andes range and the basement.

Geologic History of the Study Area

The South American continent was assembled by accretion of Proterozoic-age terrenes (ages from 1.8 -1.6 Ga) that become younger towards the west (Ibanez- Mejia et al., 2010). The basement in Araracuara consists of a series of medium-grade metamorphic rocks such as gneisses and migmatites, (PP-Mmg1 in Figure 1.2; Gómez et al., 2015). Marine transgressions deposited mudstones and sandstones during the Ordovician and Silurian Periods (O-Sm, Figure 1.2), these beds are known as the Araracuara Formation (Bogotá, 1983). Separation of Africa and South America during the breakup of supercontinent Pangaea in the Mesozoic created the Atlantic Ocean and endowed South America with a passive margin to the east and a convergent margin to the west. The subduction of the Nazca and Coco's plates under the South American plate, western margin of the continent, is responsible for the continental volcanic arc and compressional tectonics that built the Andes Cordillera (Mora et al., 2010).

During the Miocene Epoch, much of the Amazon lowland, east of the modern-day Andes, was flooded. A system of wetlands, rivers and estuaries connected to the north, with the Caribbean Sea (Hoorn et al., 2010). This flooded period included episodes of marine transgression (Hovikoski et al., 2010) that enriched the lowland clays with salts and phosphates. The sedimentary rocks formed during the Miocene are mapped as N1-Sc

in the official geological survey map (Figure 1.2, modified from Gómez et al., 2015). The N1-Sc unit appears to correspond to the Mariñame Sandstone unit (Hoorn et al. 2010) north of La Chorrera, and to the Pebas Formation (Nutall, 1990; Rasanen, et al., 1995; Ruegg & Rosenzweig, 1949) south of La Chorrera . The Late Miocene marked the beginning of considerable shortening in the Northern Andes (see Mora et al., 2010 and references therein.).

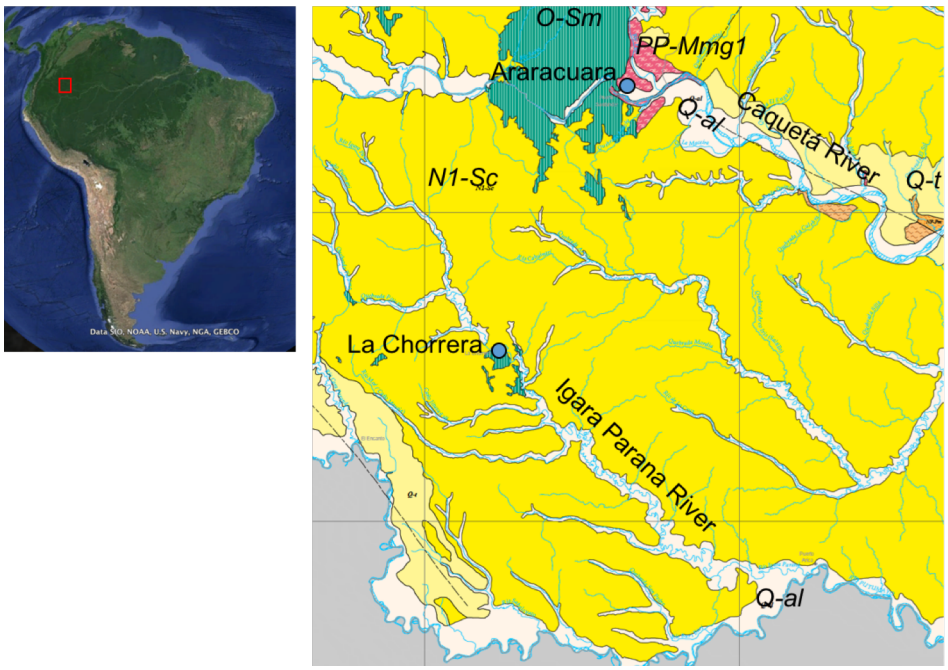

Figure 1.2. Geological map of the study area. PPMmg1: Paleo-Proterozoic medium-grade metamorphic rocks; O-Sm Ordovicic-Silurian marine rocks; N1-Sc: Neogene, Miocene Sedimentary continental rocks; Q-al: Quaternary alluvial; Q-t: Quaternary terraces. Modified from Gómez et al., 2015.

The evolution of the modern Amazon River is linked to the uplift of the Andes and basement arches. For example, the Vaupés swell formed a barrier to the north, causing the waters of the Amazon and Orinoco rivers to disperse. The modern east-

flowing Amazon system was established about 11.5 million years ago (Figuereido et al., 2009). The late Pleistocene and Holocene Epochs were marked by glacial and interglacial periods with development of glacial caps in the Andes, and sea level fluctuations. Sedimentation patterns, and erosional/depositional cycles in the Caquetá river terraces record the glaciations in the high Andes (van der Hammen et al., 1992). Archaeological evidence indicates that the first humans arrived in South America between 18,500 and 14,500 cal. before present (Dillehay et al., 2015).

The tribal territory holds different kinds of wealth: natural, environmental, and cultural. Geologically, the lands contain important natural and mineral resources, e.g., alluvial gold, clay, and huge reserves of fresh water. The landscape in Araracuara has features of geologic interest; such as the Araracuara canyon carved by the Caquetá River through Paleozoic sandstones; colorful outcrops of migmatites and granitoids (1.8 Ga, Ibanez-Mejia et al., 2010); and a system of river terraces that record the Quaternary glaciations that affected the Andes (Van der Hammen, 1992).

The intellectual and emotional meaning attached to these physical features by the Uitoto form the places of the cultural landscape. Places in the Uitoto territory, such as the *Chorro de Juma* (Juma's rapid), *Ciudad perdida* ("lost city," an area of big boulders), or *komuimafo* (the hole of awakening), are believed to have spiritual owners, who are their guardians and caretakers. Places like this form the basis of the traditional education and way of life (Echeverri, 2008). The landscape is a focal point in which geology, culture, and education converge.

Methods

Study Site

Araracuara is located in the northwest Amazon basin (latitude 0º 38´ South; longitude 72º 23´ West), and situated on the north bank of the Caquetá River in the middle part of its basin (Figures 1.1 & 1.2). The middle Caquetá area is relatively flat-lying; elevations vary between 90–280 m above sea level. It is one of the rainiest regions in the Amazon with a mean annual precipitation of 3,059 mm. A period of heavy rainfall from April to November alternates with a drier period during the rest of the year (IGAC, 2002).

Tribal Permits and Informed Consent

Permissions to conduct this research were granted by the Regional Indigenous Council for the Middle Amazonas (CRIMA) and from the government of Araracuara. The research was approved by the ASU–IRB as exempted research (see Appendix A). The informed consent was obtained from all the participants. They also gave permission to use their names. The Elder, Vicente Makuritofe, a former *capitán* and *vice-cacique* (chief and traditional authority) of Araracuara, collaborated in this study. He has been an author, co-author, and collaborator in several academic works on traditional knowledge (e.g., Garzón & Makuritofe, 1990; Makuritofe & Castro, 2008; Vasco-Palacios et al., 2008). I also worked with his extended family living in Bogotá and in the Araracuara area, in Colombia. The information presented in this paper is that of Makuritofe's clan, the *Geeia* clan.

Field Methods

Data was collected during two fieldtrips in 2006 and 2010. Methods from field geology (Compton, 1985) and field ethnography were applied.

Geological field methods were used to describe and identify hand samples of rocks and clays. Sampling locations were characterized using topographic profiles, general observations of the landscape, and geospatial referencing with a GPS receiver. The geological map used in the study was obtained from the Colombian Geological Survey (INGEOMINAS, 2007). Management and visualization of geographic data utilized ArcGIS 10 software (ESRI, 2010).

From ethnography, I used a Participatory Rapid-Assessment (PRA) approach, a participant observation method for data collection conducted in a very short period of time (Bernard, 2002). The participants, or subjects, were pre-selected and contacted based on their established expertise in Uitoto culture, which is a form of purposive sampling (Bernard, 2002). The data were collected mostly from the Elder Vicente Makuritofe, who collaborated in multiple academic works as a traditional medicine man (e.g., Garzón & Makuritofe, 1990; Henao, 1996; Makuritofe & Castro, 2008; Vasco-Palacios et al., 2008). He and his family, some of whom live in Bogotá, Colombia, were interviewed for this work.

I used unstructured and semi-structured interviews in Spanish, a language in which the Uitoto are fluent. Some information was shared in Uitoto language, and in those cases, a family member of the Elder translated it into Spanish. The ethnographic method of free listing, i.e., asking people to list all the rocks, minerals, and *gredas* (clays) that they could think of, in order to find out names for geological materials was used to obtain names of geological resources.

The field trips around Araracuara were lead by the Elder, who indicated localities and geological resources of interest in accordance with a mutual understanding of the goal of the project: to learn about relevant geological features and materials for the Uitoto. Because clay minerals are a relevant geological resource, also used in traditional medicine, and that also form most of the surface geology of the area, the properties of the clay minerals were characterized from the cultural knowledge and Western Science. To learn about sources of curative clays in the territory, the Elder's grandson mapped their location (Figure 1.3).

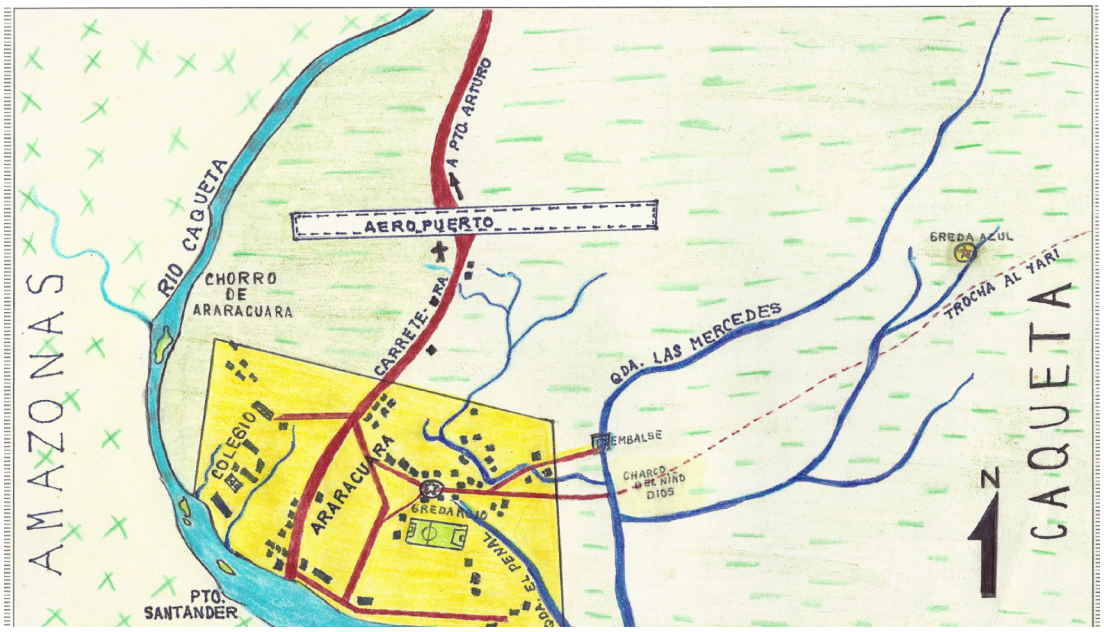

Figure 1.3. Map of Araracuara elaborated by a cultural expert. The source of blue clay used for pottery and medicinal purposes (*greda azúl)* is indicated with a star.

Laboratory analyses of clay sample. The mineralogy of the curative clays was studied using X-ray diffraction (XRD) using a Bruker D8 advance spectrometer with Soller anti-scattering slits and graphite monochromator with Ni-filter and Cu-anode radiation. Samples were analyzed as random powders and as oriented, air-dried, ethylene-glycolated sections and heated sections (500 °C following the methods described in Moore & Reynolds, 1989). Qualitative mineral analysis was performed using XPowder software (Martin, 2004).

Archival Research. Historic documents and unpublished material (thesis, dissertations) were used to inform this study.

Results and Discussion

Geological Metaphors

The mother, the machine, and Earth systems. Metaphors are used to explain and understand a phenomenon (i.e., target domain) in terms of another phenomenon that is better known (i.e., source domain; Lakoff & Johnson, 1980). The metaphors used by westerners and indigenous peoples to understand the target domain 'Earth' differ in their source domain. For example, it is common for Native people to think about the Earth in terms of a body, an organism, or a living system (Aikenhead, 1997; Cajete, 2000), while Western geologist refer to machines, and non-living systems, as source domains (Semken, 2005). Identifying the metaphors used by cultures is important because they shape the relationships between societies and the environment. Europeans thought of the environment as an object: something to be conquered, exploited, and modified in order to serve the demands and desires of men (Sioli, 1984). A different relationship is established

by cultures that consider the Earth as a living being, something to be honored, respected, and cared for (Cajete, 2000; Salmon, 2000). Conflicting metaphors can discourage scientific interests in native students (Aikenhead, 1997; Riggs & Semken, 2001; Semken 2005). It is therefore important for curriculum designers to acknowledge and use appropriate metaphors in cross-cultural science education.

The Uitoto understand their territory as the body of their mother (AZICATCH, 2006; CRIMA, 2012). Earth features and materials are parts of the body, and energy flows through them as it might in living beings (Echeverri, 2005). In the lineage of the *Geeia* clan (Makuritofe's lineage), crude oil is the blood; and metals, such as gold, are glands. The body of the territory is said to be female with two breasts, one feeds the wildlife (i.e., salt licks), and the other feeds humans (agriculture products and game); her womb is the ocean, and the amniotic fluid is the salt water. She gave birth to the tribes, according to an origin story that holds that people came to the forest through a cave, a hole, located in La Chorrera, called *Komuimafo*, the hole of awakening. Next to the cave, a muddy lake (*Uigojɨ*) was the place where the first humans rinsed off the dirt after a wasp cut their monkey tail (Urbina, 2010). The cave is a private, sacred place. It would be culturally inappropriate to propose a geological field trip to that area without consulting, and obtaining permission from traditional authorities.

Western geology does not typically consider the Earth, or any region on it, as a mother, nor does it consider it alive. Generally, mechanical metaphors are used to explain Earth's functioning in most mainstream textbooks (e.g., global conveyor belt, isotopic clock, convection cell). Geologists typically make a sharp distinction between a substrate

that is devoid of life and the biosphere that lives on top of it, whereas, indigenous researchers describe geological processes as manifestations of living systems (Aikenhead, 1997). Such conflicts between Western and Indigenous views could hinder the interest and engagement of indigenous students in Western Science (Aikenhead & Jegede, 1999; Murray, 1997; Semken, 2005). An alternative is using Earth–System Science (EES) for instruction. ESS is a mainstream scientific concept that considers the interactions between the Earth systems (e.g., hydrosphere, geosphere, atmosphere, biosphere) and the human impacts on them. I posit that ESS is compatible with the systemic and holistic way in which the Uitoto think.

Weathering: rock's excretion. The Uitoto explain certain geological materials (e.g., clayey soils) as the feces of other rocks. The meaning of this metaphor remains to be studied, but it is possible that the texture and color of certain rocks resemble feces. It could also be a metaphor for the weathering process: a parent material breaks down chemically (metaphorically, it is digested) and changes into an altered material impoverished in certain elements.

Salt licks: The mother's breast for wildlife. Salt licks are the breast of Mother Nature for wildlife, according to the Uitoto. Salt licks are often visited by animals with the purpose of licking or consuming mineral deposits enriched with nutrients (e.g., P, Ca, Na, K, Mg, and Cl) compared to adjacent soils and rocks (Montenegro, 2004). Western scientists agree that the animals supplement their diet through soil consumption (Abrahams, 2004; Diamond, 1999; Gilardi et al., 1999; Johns & Duquete, 1991; Lips & Duivenvoorden, 1991). Furthermore, visiting animals may use the mud for medicinal

purposes. Indigenous and Western researchers have observed how wounded animals cover in mud and stay in the mud for several days, until their injuries heal (Makuritofe, field notes 2012). Some of the salt licks in the Middle Caquetá area developed on the clays of the Neogene Pebas Formation (Lips & Duivenvoorden, 1991); it is possible that the therapeutic effects derive from the antibacterial clays that has been documented in the area (Londoño & Williams, 2015).

Rocks, Stones and Minerals: Friends or Foes?

As referred to the Elder, certain rocks are remnants of mythic characters, either enchanted people that were punished, or great chiefs who consecrated their bodies to nature for the benefit of future generations. Other rocky places are thought of as baskets, places that keep sickness and suffering secured. The subsurface is the world underneath, the place of primeval forces, 'beings', that should remain in their domains. Releasing the locked forces would be harmful for the environment and for people. Consequently, mining is not part of the Uitoto cultural knowledge. Yet, since the 1980's indigenous peoples in the Colombian Amazon mine the rivers in an artisanal way, a practice started by the Brazilians (*garimpeiros*) less than a century ago in the Colombian Amazon (Tropenbos, 2012).

Gold: Part of the system's energy budget. Alluvial gold occurs in the region, and although its provenance has not been determined, it probably derives from the erosion of igneous and metamorphic rocks and modern volcanic interactions extensively exposed in the headwaters of the Caquetá and Putumayo Rivers (B. Stallard, personal communication, March 30, 2016). In the Uitoto tradition gold is part of the anatomy of the Earth that should not be removed because it maintains balance.

> If a human gets his/her glands removed, he/she will get weaker, the same thing happens to Earth: the gold is like her glands, and removing them from the river debilitates the system (Makuritofe, field notes, 2013).

Other indigenous peoples of the area do not equate gold to glands, but agree that the gold regulates the energy:

> When we remove a wealth of a place, the place may go crazy, it is like an unconscious person and it can turn against its own kind. The territory starts losing the energy that sustains it (Tropenbos, 2012).

Despite these cultural beliefs, alluvial gold is currently mined along the Caquetá River. The gold rush inflated the prices for necessary staples, created degradation of environmental and human health (including mercury poisoning), increased the incidence of sexually transmitted diseases, and violence (Betancur, 2015). A traditional authority explained the afflictions along the Caquetá River as an energy loss that weakened not only the natural system but also the indigenous knowledge (Tropenbos, 2012). Contrary to gold, other resources, such as clay, have cultural uses and applications.

Clays: Allies to fight disease. Indigenous cultures throughout America knew and used clays to detoxify food or to cure certain ailments. Examples include the clay used by the Hopi to make wild potato edible (Withing, 1939 cited in Johns, 1986), clay used by Aztecs and Maya in central America in traditional medicine (Sahagún, 1985), clay as a staple, traded by Andean peoples in Perú, Bolivia, and Colombia (Browman, 2004; Gutierrez, 1985; Patiño, 1984), and curative earths used by Amazonian and Orinoquian tribes (Bueno, 1933; Gumilla, 1741; von Humboldt & Bonpland, 1956), including the Uitoto (Londoño & Williams, 2014; 2016).

We collected and studied five healing clays according to the Elder Makuritofe (in *Uitoto Ñipode*): *éyikino, eyikipo, eggopaigue, eggopakipo,* and *nóggora*. *Nóggora* has four varieties: blue/green (*mókkorede*), red (*jíairede*), black (*jĩ direde*), and white (*úterede*) (Table 1.1). Results showed that Native and Western sciences recognize the healing applications of clay, although the explanations for their effectiveness differ.

Why do clays cure? In Uitoto folk medicine, certain geologic materials can be hot or cold, a classification also used for diseases which is popular in folk medical systems in Latin America (Currier, 1966; Weller, 1986). Cold stones are used to treat hot afflictions and hot stones to treat cold diseases (Makuritofe, field notes 2006). Basically, because clay is a cold material, it can treat hot diseases, e.g., heartburn and fever (hot diseases). In Western Science, the clay's large surface area, small particle size, surface charge, and capacity to exchange ions have been advocated as important properties for their healing action (Williams et al., 2009; Williams et al., 2011).

A mixture of clay minerals compose the samples recovered, with kaolinite being the most common among them (Table 1.1). Kaolinite dominates the composition of clays ingested to relieve indigestion, get rid of toxins present in the diet, or promote wound healing in traditional medicine (Table 1.1). Kaolinite is a common ingredient in pharmaceutical products as an anti-diarrheal, dermatological protector, and anti-inflammatory; it also adsorbs toxins, bacteria, and viruses (Carretero, 2002). Smectite is another clay mineral present in three of the Amazonian samples (*éyikino*, *mókkorede nóggora*, and *úterede nóggora;* Table 1.1). Smectites can adsorb water, ions, and polar molecules in the interlayer and have been used to regulate bowel movements to detoxify

plant or animal compounds present in the diet and to deliver drugs or antibacterial metals (Carretero, 2010).

Table 1.1

Names in English and in Uitoto- Ñɨpode of Samples Recovered, Descriptions, Mineralogy and Uses in Folk Medicine.

Common Name	Name in Uitoto	Description	Qualitative Mineralogy	Reported Therapeutic Uses
Blue Clay	*Mókkorede nóggora*	Light gray, silky luster, concoidal fracture. Fish odor according to Uitoto participants.	Qz + K ++ I–I/S++	To treat stomach ache, indigestion; fever; also to treat a person with jaundice
White clay	*Úterede nóggora*	White yellowish clay, high plasticity, low permeability, fish odor, contains sand grains. Collected near a creek	Qz +++ K ++ I/S +	To treat upset stomach
Kaolinite	*Ogokɨno*	Gray-white color, contains fine-grained sand.	Hal +++ Amorph. +	Used on scrapes, scratches to promote scarification; to treat gastric ulcer
Mottled Clay	*Eggopaɨgue*	Sticky material mostly clay, color brown, high plasticity, low permeability, fish odor, bitter taste.	Qz ++ K +++ I (tr) Gibb + Ana (tr) O.M -	Ingested to counteract poisoning with toxic plants such as *Barbasco* sp. (*Jɨpaiya*) and/or Yucca Brava poison. Vomit is produced. / Antacids.
Red soil	*éyikɨno*	Muddy material that can be red, pink, purple, or white.	K+++ Qz ++ I/S + Gibb + Ana (tr)	To treat fever, stomach pain, nausea, and chills associated with malaria. Cleanses the stomach and intestines.

Note. Mineralogy: Amorph: amorphous, Ana: Anatase (TiO_2), Gibb: Gibbsite, Hal: halloysite (hydrated kaolinite), I: illite, I/S: Illite-Smectite, K: kaolinite, OM: Organic matter, Qz: quartz. Relative abundances of minerals: +++ Dominant (> 50 %), ++abundant (25–50 %), + subordinate (5–25 %), - presence (1-5%), (tr.) trace (< 1 %).

Uitoto clays used in traditional medicine.

Mókkorede nóggora is ingested by the Uitoto in small pellets to aid digestion and sometimes to treat fever. This clay has been identified as an antibacterial clay (see Chapter 3 and 4; see also Londoño & Williams, 2016).

Eggopaigue, a kaolinitic soil of the type ali-acrisol (Duivenvoorden & Lips, 1991) is used to treat intoxications with *barbasco (sp)*. The varieties of the barbasco plant: *Clibadium suranimensis* and *Lonchocarpus nicou* are known in the Colombian Amazon as 'leaf–*barbasco*' and 'root–*barbasco'*. These are ichthyotoxic legumes used for fishing (Kamen-Kaye, 1977). Rotenone, a polar alkaloid used as insecticide, is the active ingredient in these species. Deliberate ingestion of *barbasco* can be fatal (Wood et al., 2005). Kaolinite may protect the gastrointestinal tract by adhering to the gastric and intestinal mucous (Williams & Hillier, 2014), it is possible that the kaolinitic soil could prevent the body's adsorption of the rotenone molecule by adsorbing the molecule, or by saturating the adsorption sites in the intestine. These are hypotheses that deserve further study.

Models and information about the Earth and geological resources allowed the Uitoto to exploit geological resources, such as clays, for medicinal purposes. Uitoto geological knowledge is essentially unknown to mainstream geology, although both Westerners and indigenous peoples have reached similar conclusions about the application of clays in medicine. Understanding the scientific basis of Uitoto knowledge about the environment is a stepping-stone towards ethnoeducation, and a more comprehensive understanding of the Amazon rain forest.

Ethnoeducation in Colombia

Cross-cultural and multilingual education that fits the cultural characteristics, needs, and aspirations of ethnic groups is called Ethnoeducation in Colombia (Ministerio Nacional de Educación [MEN], 2001). Ethnoeducation recognizes that different cultures coexist in Colombia, and that education should serve and include them all. For this purpose, ethnoeducation needs to be tailored for, and by, the groups that it intends to serve. Thus, ethnic groups elaborate and implement the Institutional Educational Project (*Proyecto Educativo Institucional* PEI, in Spanish), which includes the pedagogic strategies, and program of studies for the tribal schools (Law 115, General Law of Education; MEN, 1994). Although the ethnoeducation approach has been criticized for leaving out fundamental questions such as what education is, or how is it pursued by different cultures (Echeverri, 2008; Mueses Delgado, 2008), ethnoeducation represents a real possibility to apply ethnogeology in education.

Implications of this Study for Ethnoducation

The Colombian Amazon is a superb outdoor classroom for natural sciences available to indigenous students. Emphasizing the local environment and including cultural knowledge are research proven strategies for Native American learners, whose cultures are rooted in place (e.g., Semken, 1997; Semken & Butler Freeman, 2008; Williams & Semken, 2011). The traditional Uitoto education is also rooted in place, as they have declared, the "**Territory is the base of education**":

> The origin of the dances, of chants, and prayers are deposited in the Territory (…) The territory guards that which has been lost, that which can be recovered, and that which should stay asleep because is not 'Word of Life', but pertains to the 'Word of destruction and sorcery' (Muinane/

> Uitoto Elder, during one of the Muinane gatherings concerning ethnoeducation, quoted in Echeverri, 2008).

Place-based educational materials with an ethnogeological approach are presented in Chapter 5 and Annex F. These units could enrich the local curricula, make science relevant for native students, enhance participation of indigenous students in Earth sciences, and encourage cultural appreciation in Colombia, as well as it has done in North America.

Conclusion

In this Chapter I explored the geological understanding of the Uitoto about their territory and identified metaphors, models, and concepts that can be used to design educational materials for tribal schools in the Colombian Amazon. This study also introduces healing clay minerals and presents the Western Science and Uitoto knowledge about the basis of medicinal applications of clays.

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CHAPTER 2

ETHNOGEOLOGY IN AMAZONIA: SURFACE-WATER SYSTEMS IN THE COLOMBIAN AMAZON, FROM PERSPECTIVES OF UITOTO TRADITIONAL KNOWLEDGE AND MAINSTREAM HYDROLOGY

Abstract

Ethnogeology, the scientific study of geological knowledge of groups such as indigenous peoples, can be combined with mainstream geological sciences to enhance our understanding of Earth systems. The Amazon rainforest has been extensively studied by both mainstream scientists and indigenous researchers. We argue that knowledge of Amazonian geology and hydrology held by Uitoto experts is valid, empirically-based, and in many cases more nuanced than mainstream scientific knowledge. We also argue that knowledge sharing between mainstream and indigenous researchers can improve geological and environmental knowledge on both sides, and provide solutions for current environmental problems such as increased pressure on water resources and global warming. We applied methods from ethnography and earth science to examine the traditional ecological knowledge of an Amazonian tribe in Colombia, the Uitoto, about water, and how that knowledge correlates with what mainstream earth scientists know. The study demonstrates how ethnogeology can be applied in a water-rich environment to: (1) compare knowledge about the natural history of an area, (2) study the geological resources available and their uses, and (3) examine the bases of native classification schemes with mainstream science methods. We found parallels and complementary

concepts in the two bodies of knowledge. Our results suggest that the Uitoto have a meticulous taxonomy for water and wetlands: knowledge that is essential to protect, conserve, and manage their water resources of Amazonia.

Introduction

Indigenous peoples acquire and pass on knowledge about their environments over hundreds of years. Their environmental knowledge is a subset of a broader system of knowledge variously referred to as traditional Ecological Knowledge Knowledge (TEK), native science, native ethnoscience, or local knowledge. The epistemological correspondence between such systems of knowledge and that of mainstream (also Western and Eurocentric) science has long been debated (e.g., Snively & Corsiglia, 2001; van Eijck & Roth, 2007; El-Hani & Bandeira, 2008; Brayboy & Castagno, 2008; Aikenhead & Michell, 2011). Regardless, traditional geological knowledge has historically served tribal peoples in many of the same ways that mainstream geology serves modern civilizations, for example, in cross-generational awareness of potential volcanic hazards by Native Americans in Cascadia (Barber & Barber, 2004), in locating subsurface metal ores in ancient China (Tang, 1984), and in managing limited water resources in the arid Puebloan lands of North America (Snead, 2006). The geological knowledge compiled by past and present indigenous societies in some localities has proven useful in modern geophysical (Ludwin & Smits, 2007) and climatological (Maldonado et al., 2014) research, and ethnogeological methods have been used to interpret cultural knowledge relevant to urban water-management policy (Gartin et al., 2010).

Ethnogeology (Kamen-Kaye, 1975; Murray, 1997; Semken, 2005) is the scientific study of relationships between peoples and Earth's systems (i.e., Earth's materials, structures, processes, resources, hazards, and history; Kamen-Kaye, 1975; Murray, 1997; Semken, 2005). It has the potential to meet challenges of cultural and environmental sustainability, particularly in contested or threatened places (e.g., Semken & Brandt, 2010). For example, the ethnogeologic study of key natural resources, such as surface water, can inform plans for conservation, protection, sustainable use, and management on tribal lands.

In this paper, we argue that indigenous geological knowledge is valid, is empirically based, and correlates with mainstream scientific knowledge, which can be enhanced by thoughtful consideration of native systems of knowledge characterized by ethnogeology. Our case study of traditional Uitoto knowledge about the tribe's water-rich ancestral lands in the Amazon rain forest provides evidence for our argument and suggests that indigenous knowledge about hydrological systems can complement Western scientific knowledge. Similarly, in this time of rapid climate change, bodies of mainstream knowledge and technology may be useful to native peoples for resource management.

Uitoto Traditional Knowledge

Traditional, specialized knowledge is orally transmitted by the Uitoto[2] in a nighttime ritual, the *mambeo* (Urbina Rangel, 1988; Echeverri, 1997), during which two

[2] Uitoto, also spelled Witoto and Huitoto, has four related dialects, one of which, *N~~ɨ~~pode*, is spoken in our study area. We adopt the spelling developed by Griffiths et al. (2001), which is endorsed by the

plant-based preparations are consumed, *mambe* and *ambil* . *Mambe* is composed of powdered coca leaves (*Erythroxylum coca*) and ash from a tree (*Cecropia sp.*). *Ambil* is tobacco paste mixed with different kinds of vegetable salts (Echeverri & Roman, 2011). Uitoto tradition holds that these two preparations, properly used, induce increased focus, memory, intelligence, and alertness.

Knowledge transmitted during the ritual covers a variety of topics, including nature and its phenomena and why the elements behave in one way and not in another (Corredor, 1986). Detailed knowledge of place is essential to peoples who make their living by foraging, fishing, and/or horticulture, and the Uitoto know their environment intimately. The cultural specialist may also address ethics and proper behavior toward oneself, others, and the environment (Urbina Rangel, 2010). The theories learned in the nighttime are practiced during the day. This is expressed as the "dawning of the word," the word spoken should be seen, become concrete, in the form of an action or product.

Traditional Annual Ecological Cycle

The Uitoto ecological calendar is based on the moon cycle; 13 full moons in a year are grouped into 12 periods (one of the periods has two full moons). The 12 periods are divided into four seasons: two summers and two winters, each with different length. Each season is characterized in terms of precipitation, temperature, plants that blossom or

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Uitoto *N̶ipode*. In the orthography of Uitoto, there is frequent use of the i vowel barred [ɨ], a high central vowel, pronounced similar to English "just" for some speakers. In this paper, we use italic and underline for words in Uitoto and italics for words in a language other than English. Proper names are not italicized.

bear fruits, animal behavior, and prevalence of diseases (Makuritofe & Castro, 2008). Here, we present the most salient characteristics of the seasons with emphasis on water resources.

Yarímona **(cicada summer).** This is the beginning of the traditional annual cycle; it roughly corresponds to October and November. Temperatures are high, and thunderstorms are common, and although rain events can last for up to 3 days, this is a relatively dry season. The river level rises and falls. Viruses, parasites, and diarrhea are common diseases at this time. This season is also known for the different worms, maggots, and grubs in the environment. Thus, it is perceived as a time of disease for both the environment and people, and certain behaviors need to be observed to prevent disease.

Pɨmóna -Uáikɨ **(summer harvest; rain).** During this season, certain palms and trees bear fruits, marking different times of the summer. This is harvesting time. The first part of the summer is the driest of the annual cycle, and the river decreases to its lowest level (December–January). The winds blow gently from the east. During March, precipitation increases and continues through April and May. The river starts to steadily rise for the first time in the annual cycle. Various water bodies interconnect, forming avenues for aquatic species. Fruits and flowers fall into the water of the flooded forest, providing food for fish and animals. Fish lay their eggs in channels. Toward the end of the season, the precipitation decreases, and temperatures increase.

Riaki **(reproduction time).** This is the time when most animals reproduce (June and July). Fruit is abundant until the beginning of the wintertime, thanks to the rain received in the previous season. The river first rises rapidly and then recedes.

Nóki-Rótti **(winter and cold time).** This season includes four lunar cycles, two of which are named after plants that bloom (green *Guacarí* and tobacco), and two of which are named after the weather (gentle rain and *friaje*. *Friaje* is a climatic phenomenon produced by the advance of a cold front and high-speed winds, which cause a temperature drop of 10 – 20°C. It is a time of strong wind, thunderstorms, and rain. The pervasive rain causes the water levels to rise, and the last annual flooding of the river occurs during this time (August and September). The flooding event is called the *Canangucho* flood because it coincides with the ripening of the fruit of the *Canangucho* (*Mauritia flexuosa)* palm, or *kínére* (*Mauritia flexuosa*). After this season, the calendar starts again.

Indigenous people of the Amazon have observed how their finely tuned traditional calendars do not predict the events as well as they formerly did (Makuritofe & Castro, 2008; Londoño, this study). Mainstream researchers have used the detailed calendars to investigate the offsets produced by climate change in the Amazon Basin (Kronik & Verner, 2010). For example, the *friaje* begins and ends earlier, it is warmer, and its winds are milder. For the indigenous people of the Amazon, the *friaje* revitalizes and cleanses the jungle, and it is in itself a diagnostic of the wellness of the forest: A weak *friaje* means a weakening of the entire ecological system (Kronic & Verner, 2010).

Methods

We applied research methods from two sciences (geology and anthropology) to conduct a case study of Uitoto traditional knowledge. We consulted with the regional and local indigenous authorities: The Regional Indigenous Council for the Middle Amazon, and the Araracuara government. We obtained and documented informed consent from all the participants involved in the study. The study was approved by the Institutional Review Board of Arizona State University and complies with the Research Ethics guidelines of the National University of Colombia.

Study Site

The Amazon Basin has a tropical-equatorial climate. Mean temperatures range between 24 °C and 26 °C, with annual precipitation between 2500 and 3000 mm (IGAC, 2002). Precipitation shows a peak between March and May and a dry season between January and February (Corpoamazonia, 2008). The climatic region is tropical rain forest, the Thornwaite humidity index is 61%–80%, and the relative humidity can reach 85% (IGAC, 2002). Our study site is Araracuara, Colombia, located on the south bank of the Caquetá River (Figure 2.1; Japurá River in Brazil), a main tributary of the Amazon.

Selecting Participants and Indigenous Co-researchers

To learn from the Uitoto, environmental knowledge, we used Expert sampling, a kind of purposive sampling (Bernard, 2002). Purposive sampling is a non-probabilistic technique in which a particular characteristic of the population is selected according to the purpose of the study. In this case, we found two Uitoto Elders, renowned cultural specialists: the late Vicente Makuritofe and Marceliano Guerrero. Acknowledging that

the Elder Vicente Makuritofe contributed most of the content of this manuscript, he appears as a coauthor in this work. He and five of his extended family members participated actively in this research.

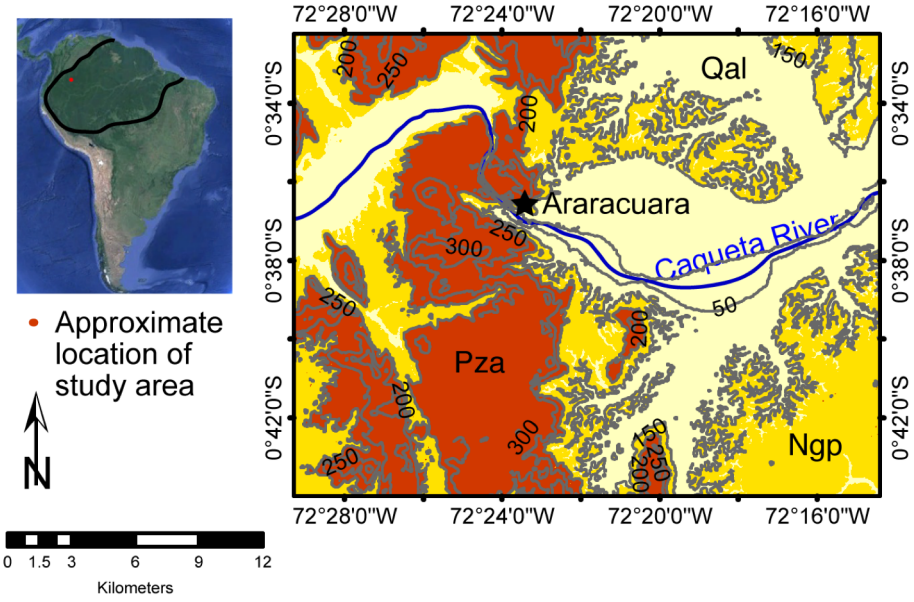

Figure 2.1. Location and geology of the area of study. Araracuara is located on the north bank of the Caquetá River in Colombia, northwest Amazon Basin. Geology: Qal : Quaternary alluvial, Ngp: Neogene (Eocene-Miocene) Pebas Formation, Pza: Paleozoic Araracuara Formation. Figure is after: Geologic Map of Colombia (INGEOMINAS, 2007).

To study traditional knowledge about water and to collect samples, we conducted field research during the months of January 2012 and August 2014. In between field seasons over the course of two years, we interviewed our collaborators in Araracuara, Bogotá and over the phone using unstructured and structured interviews (Bernard et al., 1986). To collect ethnographic data in Araracuara, we used a participatory rapid-assessment (PRA), as described in Bernard (2002). We collected stories related to rivers

or water bodies that could code environmental information. In the field area, the native co-researchers guided the field trips and draw maps of their territory and the rivers or water bodies important to them (Figure 2.2). Native researchers conducted field research in August 2014, they collected and sent water samples and were interviewed over the phone.

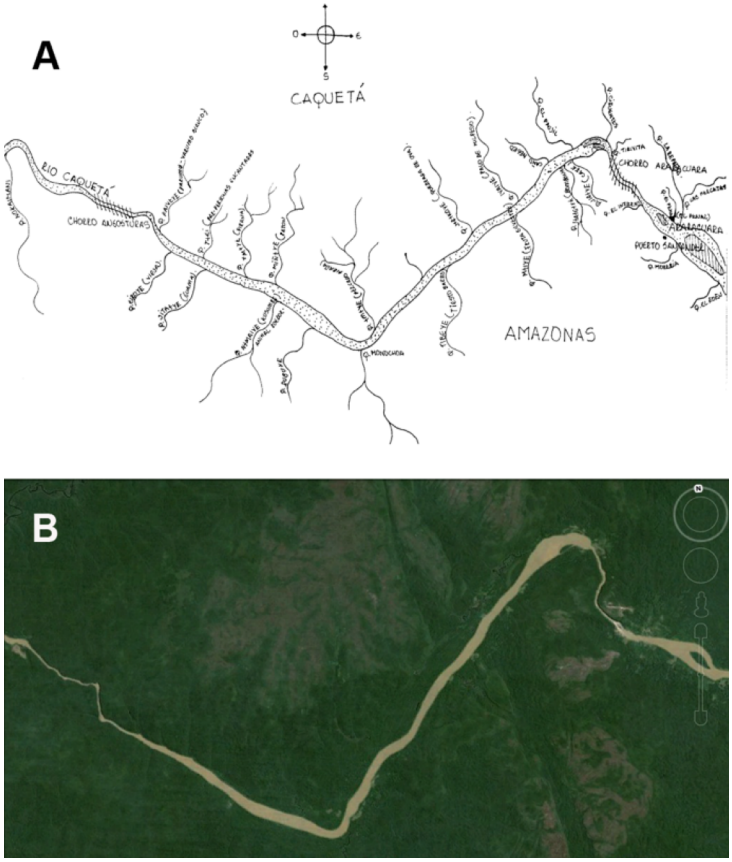

Figure 2.2. Participatory mapping can produce maps that are comparable to satellite imagery or official cartography, but contain more local information. A. Map showing the names of the rivers between Angosturas and Araracuara canyon with their names in Uitoto *Nipode* and Spanish. The location of Caquetá and Amazonas Departments (equivalent to States) are shown. Drawn by Vicente Hernandez, Uitoto researcher. B. Landsat image of the same area showing the detail of river morphology attained by the author.

Water Sampling and Analysis

The water samples were collected in August 2014, when the main flood (*canangucho* flood) was receding but water levels were still high. Samples were located on a map by native (Hernandez M. & Hernandez T.) and non-native scientists (Londoño & Garzón); the approximate location of sample sites is shown in Figure 2.3.

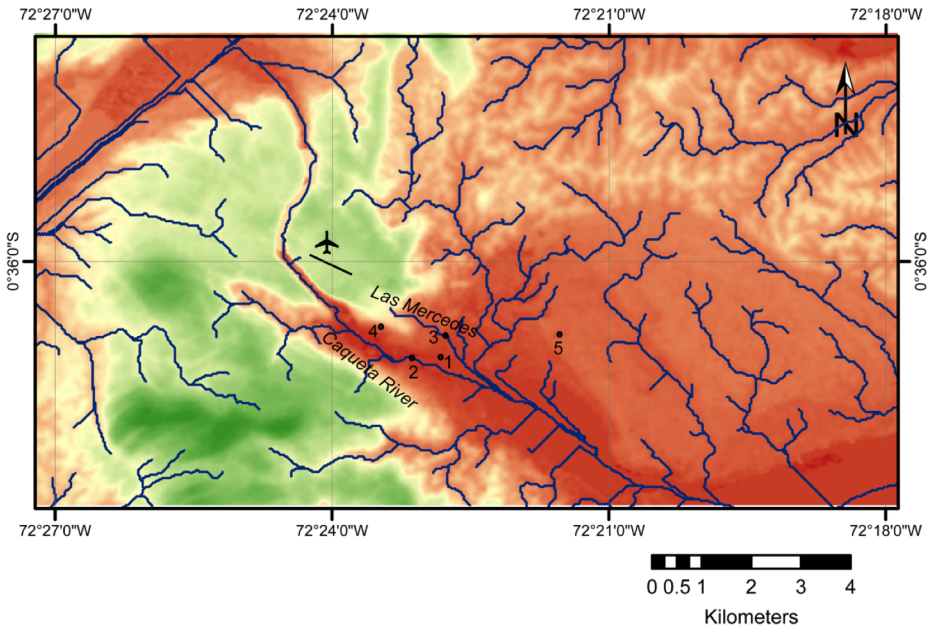

Figure 2.3. Hydrology map of Araracuara and location of sampling sites. 1. *Chururapo* (spring), 2. *imáni* (main river), 3. *jíruebɨ* (muddy water), 4. *jɨ dɨye* (black water), 5. *kɨnére* (*Mauritia flexuosa* palm swamp). Sampling site locations are approximate (see text for details). The drainage network was generated from a DEM using TauDEM (Tarboton, 2003) and ArcGIS and Arc Map (ESRI, 2010).

To test if the water samples pertain to different western science categories, we analyzed the chemistry of the water and performed statistical analysis to identify the underlying structure of the data. One liter aliquots of water were collected in high-density polyethylene bottles. Each type of water was sampled in triplicate, for a total of

15 samples (15 L). The native researchers measured the pH of the water in the field using a colorimetric test, and wrote the value on the bottle with permanent ink. They also include the date, name of the water in Uitoto *Nipode* and in Spanish, if applicable. The samples were kept in the shade and air-shipped to Bogotá in a cargo plane within two weeks. Upon receipt, the pH and Eh of samples were measured. Samples were filtered through two syringe filters (1 μm and 0.2 μm) into two 100 mL high-density polyethylene bottles, one to measure cations and one to measure anions. The prepared samples were preserved cold.

Laboratory analyses of the water samples were conducted at Arizona State University. The concentration of major ions was determined by means of ion chromatography. Undiluted samples were run in a DIONEX DX 600 IC system. The ions measured were F^-, Cl^-, NO_2^-, Br^-, NO_3^-, SO_4^{2-}, and PO_4^{3-}. The cations measured were Na^+, K^+, NH_4^+, Mg^{2+}, and Ca^{2+}. Blanks were included. Principal component analysis (PCA) was performed using R software (R core team, 2013) on 15 water samples.

Hydrology

We mapped the surface runoff with digital elevation models (DEMs, 90 m resolution) and geographic information systems. Stream maps were generated using TauDEM tools (Tarboton, 2003) in ArcMap (ESRI, 2011), Figure 2.3

Table 2.1

Field and Laboratory Descriptions of Five Water Types

Uitoto	English	Field description (provided by co-researchers)	Lab description	pH	Cond. (mS)
Chururapo	Spring	Water comes from a spring, a hole in the dirt. The terrain does not have stones or rocks, there is no root mat, and the water comes from the dirt (*tierra*), not from clay. The water is crystalline, and is cold.	Clear water with high transparency and mud odor. Dark particles at the bottom of the bottle.	5.3	11.1
Imáni	Big River	The water was collected from the Caquetá river (*Uígonamani*). The river brings all the dirt from the cordillera.	Yellowish, medium transparency. Clay-sized particles and white particles in suspension.	6.7	19.5
Jíruebɨ	Muddy water	This water comes from a muddy place that has red, acid mud (*ellikie*); the mud is used for medicinal purposes. The water flows out of the mud, is a small spring. The water gets filtered in that mud and is good to drink. Collected near the boarding school.	Clear water associated with red mud. Odorless.	5.4	18.6
Jɨ¾dɨye	Black water	This water was collected from a small dam on a black water creek (*Las Mercedes*). The creek starts in the rocks, boulders, near the airplane strip, but the source of this water is the rain. This type of black water picks up all that is in the environment, vegetal material, the dirt that comes from the trees. It is not good to drink but it can be used for allergies on the skin.	Light yellow with yellow particles in suspension likely from plant origin. Earthy smell.	5	10
Kɨnére	Palm swamp (*Mauritia flexuosa*)	Water from a *cananguchal* (palm swamp) about a day walk from the village. The water is clear. The *cananguchal* is big, long, there are plenty of animals. There are no creeks around. the forest is high around it.	Clear water. Yellow particles in suspension of vegetal origin. The sample contained a seedling that was identified as *Mauritia flexuosa*.	5.5	9.76

Results and Discussion

The Birth of the Amazon: Uitoto Oral History Versus Mainstream Geoscience

The predominant scientific model of the origin and evolution of the Amazon River was comprehensively presented in the book *Amazonia: Landscape and Species Evolution,* edited by Horn & Wesselingh (2010), and is briefly summarized here. Before the Miocene Epoch, the topography and structure of the Amazonian craton (Archean to Mesoproterozoic; Kroonenberg & de Roever, 2010) controlled the fluvial systems of western Amazonia. During the early Miocene (~23-16 Ma), the cratonic rivers were replaced by lakes, swamps, tidal channels, and a marginal marine embayment in the north (Figure 2.4A). Continued uplift of the Northern Andes sealed the connection of the lowland with the Pacific and the Caribbean. During the late Miocene (~11 to 7 Ma), the hydrology of the Amazon Basin was dominated in the east by rivers and tides and in the west by lakes and wetlands, forming a complex environment that supported considerable biodiversity (Figure 2.4B). From 11.3 Ma onwards, Andean sediments reached the Atlantic and built the Amazon delta. The transcontinental Amazon system as we know it today was probably established around seven Ma, and is represented in Figure 2.4C (Hoorn et al., 1995; Figuereido et al., 2009).

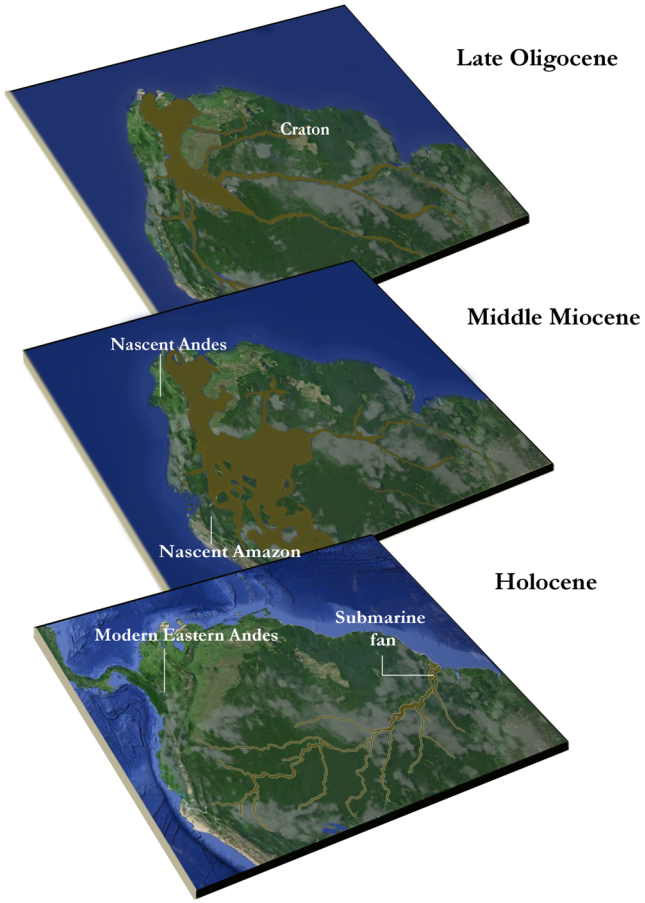

Figure 2.4. Representation of the geologic evolution the Amazon River encompassing the evolution of the landscape and species. A. In the early Miocene, the northern Andes were not uplifted. The Amazon craton topographically controlled the Amazonian drainage, and the main flow direction was towards the north and northwest. B. The development of a mega-wetland in the middle Miocene was a consequence of the nascent northern Andes that forced water down their eastern slopes and close the NW outlet of water. C. The Amazon river today flows towards the east, connecting the Andes and the Atlantic Ocean. Figure is adapted from from: Hoorn, 2006.

The Uitoto story of the origin of the Amazon has also been referenced in the literature (Urbina Rangel, 1988; Garzón & Makuritofe, 1990; Preuss, 1994; Henao, 1996; Urbina, 2010). Preuss (1994) collected an extensive corpus of Uitoto folktales, which have provided the basis for later linguistic and cultural analyses (Petersen de Piñeros,

1994). We collected the story and present here an abbreviated version. The story is also depicted graphically in Figure 2.5.

> That River was a tree, the *Monίya aména*, tree of abundance. The tree had a lake at its base that grew along with the tree. The tree produced a great variety of fruits and foods. Its abundance was so great that it produced plenty to feed all the peoples; the animals were the people of that time. However, the tree and the lake continued to grow, the tree reached impossible heights, and the lake covered vast areas. Even the flying and the swimming animals struggled to reach the food. At that point the animals agreed that, if they were to eat, they had to chop down the tree. When the animals were cutting it, the splinters and chips of wood formed different fish. From the bark came the octopus, the pink dolphin, the sea cow (manatee), and others. When the tree fell, its trunk formed the *Monίyanamani* (Amazon River), the tree of abundance. The branches formed the main tributaries, and small twigs formed creeks and gullies (Narrated by V. Makuritofe; Londoño, unpublished field notes, 2012).

Most native stories represent scientific thought and its applications metaphorically (Cajete, 2000). The tree of abundance is such a metaphorical representation and a crosscutting theme in Uitoto knowledge. Fully narrated as a chant, the story requires several nights to complete. The myth has been shown to code information for plant evolution and taxonomy (Garzón & Makuritofe, 1990), social evolution and agriculture (Henao, 1996), and the evolution of the Amazon basin (Garzón & Makuritofe, 1990; Urbina, 2010). This study is the first to reference the story to geoscience. We presume that other taxonomies of natural systems and processes (e.g., fluvial systems, animal co-evolution) may also be embedded in the *Monίya aména* narrative.

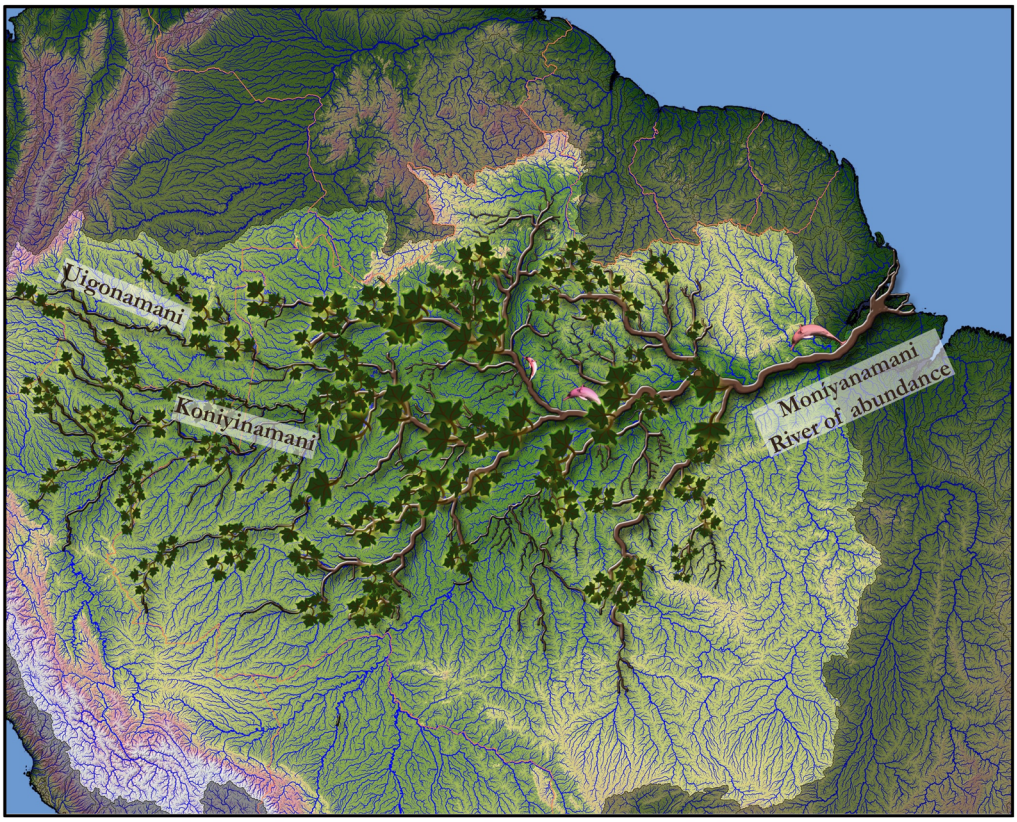

Figure 2.5. Representation of the *Moníya aména* story: Uitoto metaphor that explains the Amazon River system as a big tree that was felled and imprinted its shape on the land. The *Uigonamani* (Caquetá River) and *Koniy+namani* (Putumayo River) are main branches of the tree. Animals like the pink dolphin came out from its trunk.

Several useful parallels between mainstream geology and the *Moníya aména* narrative can be drawn, suggesting that this story could be particularly useful for place-based and culturally informed geoscience teaching. The principal points of commonality are:

(1) Amazonia was partially flooded. Mainstream geoscientists agree that during the middle to late Miocene (16-11 Ma), western Amazonia was occupied by a "mega wetland" (Hoorn et al., 2010). In the *Moníya aména* story, an immense lake

extended at the base of the tree; the *Monίyamena jórai* was "a very big lake, like a sea, that will later form the rivers and lakes of the entire region" (Garzón & Makuritofe, 1990). In other words, the mega-wetland appears to correspond to the *Monίyamena jórai.*

(2) The flooded area in western Amazonia evolved into a river system that resembles a tree. Tectonic uplift of the northern Andes ended the flood and redirected the flow of water into an interconnected drainage system. In geomorphology, the tree-shaped drainage networks are called arborescent or dendritic. In the Uitoto story, the felling of a colossal tree put an end to the lake and imprinted a tree shape onto the land.

(3) The Amazon is a biodiverse and interconnected ecosystem. The species that occurred along the development of the Amazon River have been studied by mainstream palynologists, paleobotanists, paleontologists, and molecular phylogeneticists (Hoorn & Wesselingh, 2010). The Uitoto "Tree of Abundance" story explains how animals and plants diversified and co-evolved with the landscape. Many animals originated from parts of this biodiverse tree: octopus from the root, massive aquatic animals from the bark and trunk, fish from wood splinters and from fruits. An evolutionary pattern or explanation seems to be engraved in the structure of the story. However, at a more basic level, we posit that the *Monίya aména* story would be an appropriate framework upon which to organize curricula for place-based teaching of the geology and natural history of the Amazon basin. The story provides local and highly relevant cultural context for teaching mainstream scientific concepts related to the Amazon basin.

(4) This same approach has been used successfully to teach geoscience in other indigenous communities (e.g., Semken & Morgan, 1997; Semken, 2005).

Mainstream natural scientists from diverse disciplines including geology, biology, botany, climatology, and ecology have only recently begun to collaborate across disciplines in order to better understand the evolution of Amazonia. Indigenous scientists have long used a strongly holistic (transdisciplinary) approach in order to interpret the intricate Earth system interrelationships within their home environments and landscapes, and to understand how those interrelationships influence the lives of their communities (Cajete, 2000). This is an appropriate time for mainstream and Native scientists to ally in our common pursuit of better understanding of, and more sustainable coexistence with, the global Earth system.

Water Taxonomy

Schemes used by indigenous and local peoples to classify water features in the landscape are diverse, especially in a water-rich environment such as the rain forest. In the Amazon Basin, rivers are classified by color. The color-based names of Brazilian rivers (e.g., Rio Preto [black], Rio Claro [clear], Rio Branco [white], and Rio Verde [green]) may be Portuguese adaptations of a pre-existing native nomenclature (Junk et al., 2010). Wallace, a British explorer and naturalist, introduced the color-based classification for Amazonian rivers into mainstream science (Wallace, 1889). He classified the rivers as "the white-water rivers, the blue-water rivers, and the black-water rivers". It seems likely that he borrowed these classifications from the Portuguese names. Sioli (1956; 1984) popularized Wallace's three-color classification scheme. Whitewater

rivers originate in the Andes and transport nutrient-rich sediments. Blackwater rivers are born in the Guiana shield: Their suspended load is poor in nutrients and high in humic acids and organic compounds (Duncan & Fernandes, 2010). Clearwater rivers drain the Central Brazilian shield and are only found in the Middle Amazon (Junk et al., 2011.)

Like hydrologists, the Uitoto classify the water by colors. They attribute a river's distinct color from to the "rocks and mud" present at its source and along its course. Native classifications include the colors of the animals that live in and along the rivers; it is more detailed than the standard Eurocentric classification.

> The large rivers in Colombia, Amazonas (*Moníyanamani*), Caquetá (*Uigonamani*), and Putumayo (*Koniy~~i~~namani*), are from ash waters (*Uigog~~i~~n* - *uigorende*). Mud-colored animals inhabit them. White water or white creeks (*Utegin*-*Uteye, Nogora*) are crystalline waters; they are very rare because they are born of white land and rocks (*Ogon~~i~~e*), and come with white sand. Gray-mud water (*Girueg~~i~~n*) and brown water (*Git~~i~~gin*) come from brown ground (*Git~~i~~n~~i~~ene*). Everything produced in that water—plants, fish, frogs and crickets—all are brown. Red waters (darker variety) (*Giayen- giagin*) are born from reddish lands; green waters (*Mocogin - mocoye*) emerge from green or blue lands and they also have their corresponding-colored animals and beings. It is necessary to know where the creeks or small rivers come from--that is what makes them different; for example, red (bright and intense red) waters (*Ecogin- ecolege*) are very scarce in the territory, because they are of tigers, they are not born on earth and come from the history of peoples from *Moo – Buinaima,* from *Muina~~i~~* world (Elder Marceliano Guerrero Jekone, Garzon, Unpublished field notes, 2014).

The Uitoto names for certain water bodies describe the characteristics recognized by mainstream science. For example, the Amazon is the *Moniyanamani*, (from *monie*: abundance, also to have food in abundance, and *imáni*: large/main river). Thus, the name translates as "large river of abundance," alluding both to the abundance of species and of nutrients (food) particular to the river. For the Uitoto in Araracuara, the Caquetá River is

the *Uigonamani**;** uigo**:*** dirt, thus the “large muddy river.” The Putumayo is the *Koniy~~i~~namani*, or the “large sandy river.” This name not only refers to the load but to the extensive sand beaches that the Putumayo River forms. In other words, the Uitoto names refer to characteristics that are salient to both indigenous and mainstream observers.

Not all the classification schemes of native science correspond to those of mainstream science. Native management of water is based on classifications ranging from mythical discourse to socioeconomic, environmental, and medicinal applications. For example, Uitoto stories about the "river of creation" (*Komuiya Namani)*, the source of surface and groundwater, is another way of classifying water. This classification includes categories such as *Nof~~i~~dai namani:* stone water, *zaf~~i~~dai namani:* water of sands, which is medicinal and sacred; *erig~~i~~namani*: bitter water; *rochidai namani*: acidic water; *jucure namani:* poisonous water; *jit~~i~~rue namani:* black water; *nonok~~i~~namani*: achiote (*Bixa orellana*) water; *m~~i~~k~~i~~g~~i~~i namani*: harmful water. The Uitoto describe and characterize each water body in their territory according to intricate traditional knowledge that mainstream scientists do not yet understand. This paper offers an introduction to some of that knowledge, which could be compared to and integrated with mainstream scientific understanding to increase our knowledge about the hydrology, geology, and ecology of the Amazon Basin.

Place-Based Water Names: Towards a Refined Classification for Wetlands

The Amazon basin is one of the largest wetlands in the world (Fraser & Keddy, 2005). Wetlands are natural or artificial extensions of land saturated with water either permanently or seasonally, and they provide crucial environmental services but are being

degraded rapidly; they need to be protected and used wisely (Ramsar Convention Secreataiar, 2013). Mainstream scientists (Junk et al., 2011) have only recently classified the wetlands of central and eastern Amazonia from an ecological perspective. The classification by Junk et al. (2011) integrates folk names and concepts that met the researchers' ecological criteria. In western Amazonia, remote sensing might not be able to provide the resolution necessary to study the wetlands of the western basin, especially as access to certain areas of the basin is difficult due to political and natural constraints. Indigenous knowledge could provide us with the information we need to understand and classify these wetlands.

With further study, we should be able to elucidate the Uitoto taxonomy for water and wetlands in the western basin and use it to complement our current knowledge. Below are examples of overlapping wetlands categories: first those provided by Junk et al. (2011), followed by the native experts' categories.

1. Wetlands with relatively stable water levels > Forested swamps in the rainforest palm swamps and mixed forests. These areas remain waterlogged and could be flooded during the rainy season. During the dry season, they store water and gently release it to feed connected streams. They buffer surface run-off during heavy rainstorms. Organic matter accumulates but also decomposes

***Kɨnére* (*Cananguchо* palm swamp/ *cananguchal*).** Water that originates in flooded palms forests of *cananguchо, Mauritia flexuosa*) palm. The water flows very slowly and never dry up, but its area shrinks during the dry season. The Uitoto distinguish two types of water according to the hydrological cycles of high and low water

levels. Water from low water occupies less volume and is referred to as small *cananguchal* water. It accumulates between the roots of the palms. It is grayish and smells like animal musk. The water tastes semi-acid due to the palm's biological processes, which determines its characteristics. Water from the high water season occupies a larger area and is different than the water in contact with the roots. This water is referred to as "long or large *cananguchal* water". Contrary to the small variety, the water appears clear or blue/green, has no odor and an earthy taste which some people find more palatable than the smaller *canangucho* water. It is also safe to drink.

It is a cultural belief that water bodies have owners; the owners are the caretakers of the place and its beings. For example, the black boa is the owner of the *cananguchal*; its presence guarantees that the *cananguchal* will not dry. Like the boa, the *canangucho* palm is closely associated with water and acts as a protector. This culturally- based assertion has not been tested by mainstream science, but it could indicate testable ecological interrelationships.

2. Wetlands with oscillating water levels > subjected to predictable, long-lasting, monomodal flood pulses > low flood amplitudes > hydromorphic edaphic savannas of low fertility. Areas with insufficient drainage can host interfluvial wetlands during rainy seasons. For example, strongly leached, low-fertility soils with an underlying hardpan of deposited minerals can be shallowly flooded. Depressions may be filled with fine-grained kaolinite. These places can host unique plant and animal communities. Other names for this kind of wetland in central and eastern Amazonia are

campina, bana, muri scrub vegetation*, campirana* forest*,* and *varillales.* These wetlands are difficult to access, hindering their study.

Tapíre. These places are also known locally as *chuquiales,* composed of low, dense root-mat forests. Some of the *chuquiales* are white, like a white-sand beach (*koniyiki*). The soil is sometimes soft due to the *úterede noggora* (kaolinite). Roots and branches intertwine in these areas, which are not like a lake or well, but the soil is soft (*toórede*). The water comes from rain, and the *chuquial* can dry during summer. Some of the vines provide water too, but the water is bitter (*érɨji*) and sluggish. It smells like rotten leaves and is unsafe to drink. It contains parasites or small animals. The water almost does not flow. There are beasts, dangerous animals, and it is not a good place to visit (Londoño, Makuritofe, this work).

As the examples above demonstrate, native classifications encompass ecological associations that could help to inventory and differentiate wetlands in the northwestern part of the basin, providing a framework to manage or protect them.

Chemical Analysis of Some Uitoto Water (*Jaɨnoi*) Types

Quantitative and analytic methods from mainstream science can be applied in ethnogeology. Native researchers sampled different water types: *chururapo*, *imáni, jíruebɨ, jɨ dɨye,* and *kɨnére,* translated as spring (literally: flowing from a hole), main river, muddy water black water, and palm swamp water (*Mauritia flexuosa* swamp; Table 2.1). Because the chemical distribution in streams has been used to differentiate water types in the Amazon (Gibbs, 1972; Sioli, 1984; Stallard & Edmond, 1983; Devol et al., 1995), we evaluated the ions present in the samples provided.

Rigorous water sampling for chemical analysis requires that samples are filtered-sterilized *in situ* and stored at 4°C. This preserves elements in solution and hinders biological processes, such as microbial metabolism, that utilize elements. Our samples were stabilized in Bogotá, after ~1-2 weeks of collection. Therefore, water data for N, S, and P was not considered as these elements actively participate in biochemical cycles. Conversely, the abundance of major ions, in natural waters, overwhelms the fraction that participates in biological cycles and it can be assumed that their concentration does not significantly change in the sample (conservative species). Major ions also stay in solution for longer times (up to 42 d) and are less sensitive to temperature effects (Jackson, 2000). Furthermore, the values we found for major ions correspond to results in the literature (Devol et al., 1995). Therefore, we based our analyses and conclusions on the conservative species (i.e., Na^+, K^+, Mg^{2+}, Ca^{2+} and Cl^-; Table 2.2).

The *imáni*, main river water, presents the highest ion concentration. The water with the lowest ion concentration was the *ji-diye*, black water in Uitoto. The concentration of individual ions varied in the analyzed samples; in general, *jíruebi* was the saltiest (Na^+, Cl^-), while *imáni* was rich in Ca and Mg. As described by Sioli (1956), white water is dominated by the alkaline earths (Ca^{2+} and Mg^{2+}) and the CO_3^- anion, which contributes most of the negative charge in the water of the Amazon's main stem (Stallard & Edmond, 1983).

Imáni means big or main river; it relates to the suffix *namani* found in the Uitoto names to rivers with considerable discharge, such as the Putumayo (*Koniyinamani*),

Caquetá (*Uigonamani*), and Amazonas (*Moníyanamani*), with Andean origin. Thus, at least preliminary, whitewater rivers seem to relate to the Uitoto categories *imáni*.

Traditional ways of classifying resources are based on their uses in medicine. Medical uses of Amazon waters are yet to be studied in ethnohydrology. Mainstream science has characterized black-water rivers by its organic, humid acid content. We did not measure organics in our research, but the Uitoto use the category black water. We speculate that the mainstream and native categories overlap. However, in traditional medicine, the Uitoto know of different types of medicinal black water, for example, one to treat chills, another one to treat allergies. It is possible that the documented astringent properties of the tannins present in the water contribute to its healing effects. Yet, more work is needed to learn from the elders about these advanced, and specialized topics in native science.

Jíruebɨ contains a red mud with ascribed healing properties. Antibacterial clays have been identified in the study area (Londoño & Williams, 2016), but the red mud and the water associated with it have yet to be studied.

These results show that while certain categories of water, and its properties, can be compared between Uitoto and mainstream sciences, others are unknown. In particular, the medicinal uses of water remain limited to a cultural practice. Deeper understanding of water properties could have implications for health and would provide a basis on which to propose alternative ways to value and use the water resource.

Table 2.2.

Concentration of Major Ions (μM) in Water Samples

Sample	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	NO_3^-
Chururapo-	41.4	5.6	1.1	5.3	32.9	14.5
Chururapo-	39.7	6.1	1.4	5	34.4	67.8
Chururapo-	42.2	5.9	0.8	4.5	35.8	64.2
Mean	41.1	5.9	1.1	4.9	34.4	48.8
S.D.	1.2	0.2	0.3	0.4	1.5	29.8
Imáni-1	46.4	42.1	20.4	63.4	45.5	19.3
Imáni-2	43.1	23.4	19.8	55.7	17.5	98.8
Imáni-3	40.7	19.9	19.7	56.2	10.3	10
Mean	43.4	28.4	20	58.4	24.4	42.7
S.D.	2.8	11.9	0.4	4.3	18.6	48.8
Jíruebɨ -1	42.4	8	1.1	3.3	55.5	9.9
Jíruebɨ -2	37.9	8.3	1.4	3.9	49.6	5.9
Jíruebɨ-3	63.3	9.3	0.8	3.1	63.1	6.4
Mean	47.9	8.5	1.1	3.4	56	7.4
S.D.	13.5	0.7	0.3	0.4	6.7	2.2
Jɨ̄ dɨye -1	9.6	8.2	3.1	9	5.6	43
Ji–dɨye -2	10.5	8.8	3.6	12.6	5	25
Jɨ̄ dɨye -3	10	8	3	5.8	5.7	32.5
Mean	10	8.3	3.2	9.2	5.4	33.5
S.D.	0.4	0.4	0.3	3.4	0.4	9
Kɨnére -1	23.3	7.9	5.5	15.9	14.4	3.4
Kɨnére -2	22.7	8	5.5	15.7	14.3	2.6
Kɨnére -3	22.78	7.6	5.4	15.9	13.5	2.2
Mean	22.9	7.8	5.5	15.9	14.1	2.7
S.D.	0.3	0.2	0.1	0.1	0.4	0.6

Note. NO_3^- data should be interpreted carefully due to sampling limitations. NH_4^+, F^-, NO_2^-, Br^-, SO_4^{2-} and PO_4^{3-} are below detection limits. See text for details. *Chururapo*—spring, *Imáni*—main river, *Jíruebɨ*—muddy water, *Ji—dɨye*—black water, and *Kɨnére*—*Mauritia flexuosa* palm swamp.

Principal Component Analysis (PCA)

To further analyze the water chemistry, we used principal component analysis, a statistical technique to identify the underlying structure of data. In our analysis we used the concentrations of the major ions Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-. The PCA showed that each of the native water types is distinct from every other, confirming a chemical basis for the indigenous characterizations (Figure 2.6).

In Figure 2.6, the new components, PC1 and PC2, explain 63% and 31% of the variation in the data, respectively. However, samples that plot close to the origin of the vectors (e.g., *kɨnére*) are not satisfactorily explained by the components, presumably because major ion content is insufficient to characterize the water. According to the Uitoto, *kɨnére* water cannot be separated from the *cananguchо* palm, it so closely associated that the water sample was sent with a small *cananguchо* seedling (despite the instructions of avoid sediment or any other particulates in the bottles). Because we did not collect organic data, which seems key in this case, *kɨnére* is excluded from this discussion. To the left of the X axis (PC1), the *imáni,* main river water, exemplifies waters that drain the Andes (white water). To the far right, the *jɨ dɨye,* black water, drains local lowland materials and its color relates to organic input. Thus, PC1 axis would seem to correspond to mainstream scientific classifications of watercolor. However, *jíruebɨ* and *chururapo* are not from runoff. They plot close to one another and midway between the white and black waters. Their sources are local deposits such as the Caquetá River deposits and the Miocene Pebas Formation (Figure 2.3).

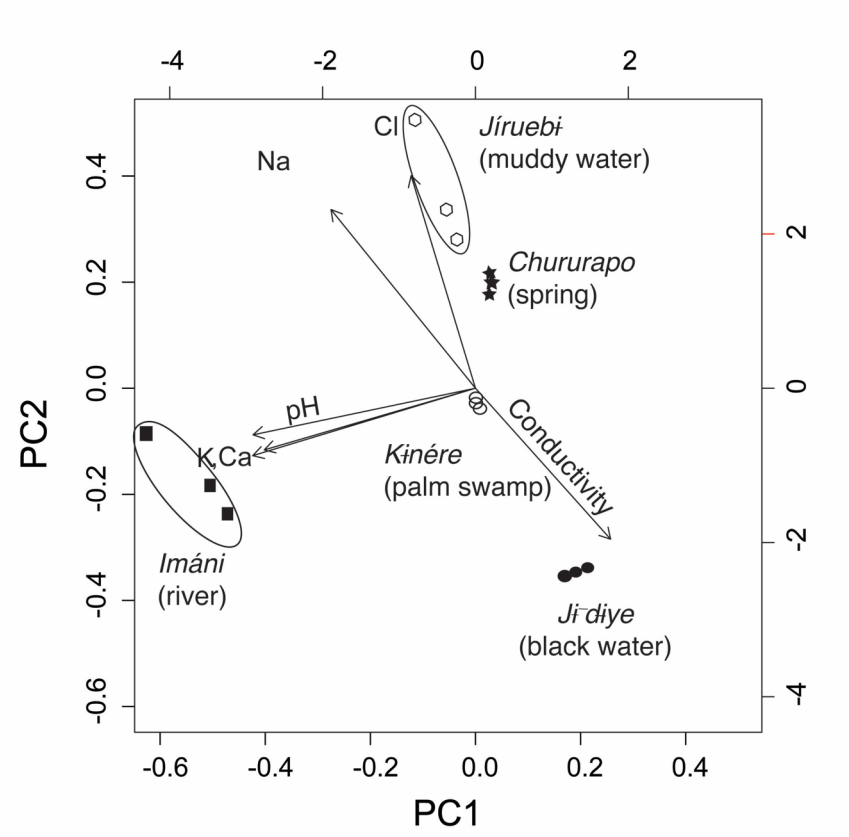

Figure 2.6. Principal component analysis of 15 water samples. –PC1 explains 63 % of the data variation and is related to water origin. PC2 explains 31% of data variation and seems to relate to the flow rate of the water.

The red mud associated with *jíruebɨ* is likely derived from soils of the Ali-acrisol group, developed in Andean-origin deposits (Lips & Duivenvoorden, 1996). However, the intense weathering environment has imprinted its geochemical signature on the Andean sediments, increasing the kaolinite, oxide and hydroxide content. Thus, the PC1 seems to represent the chemical print of materials in contact with the water: Andean to

the far left; local, weathered materials from the Andes in the center; and local, humic and organic materials in the far right; this component explains 60 % of the data.

We hypothesize that the PC2 axis in Figure 2.6 represents whether the water is running or environmentally contained. This would explain 30% of sample variation. The water from runoff (i.e. *imáni*: river, and *jɨ dɨye*: creek) plots at the bottom. The *kɨnére*, which slowly flows in the floor of the flooded forest, plots in the middle. The flow in the palm swamp is more tortuous, as it moves through a rough surface with roots, woods, and flooded-forest vegetation. At the top, *chururapo* and *jɨruebɨ* are sourced by springs. *Jɨruebɨ* water is found in red clayey soils that give the water its color. But, the swampy area is fed by a spring, not by precipitation, according to the native specialists. Therefore, it is possible that both *chururapo* and *jɨruebɨ* represent groundwater flow, or water that was environmentally contained. As we collect more data we will be able to test our hypothesis.

These chemical and statistical analyses demonstrate that the water types classified by the Uitoto are indeed different from one another, and show how the Uitoto categories can be evaluated using mainstream scientific methods.

Implications of the Study

The oral tradition is a medium to explain, conserve, and convey cultural patterns, from codes of behavior to the systematic and nomenclature that supports specific knowledge. Some oral stories are reservoirs of information that could be explored for environmental and geological data. Further study could provide a refined classification

of wetlands for the Colombian Amazon, which is necessary to enable Colombia to comply with the Ramsar convention on wetlands (IUCN, 1971; Ramsar Convention Secretariat, 2013). Findings from our study can be used to create cross-cultural educational materials for use in both native and mainstream schools.

Conclusion

We performed an ethnogeology case study with native co-researchers on the territory of the Uitoto. We compared the native knowledge of the natural history of the Amazon and the kinds, characteristics, and uses of its water with data obtained by mainstream geoscientists, and found correlations between the two. We also found that in many cases, native knowledge was more complete and nuanced than that of mainstream science. This implies that native knowledge that accrues in a given study area can enhance mainstream scientific understanding of that area. Our study demonstrates that ethnogeology can be used to conduct basic and applied research at the same time, producing both intellectual and practical outcomes.

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CHAPTER 3

UNRAVELING THE ANTIBACTERIAL MODE OF ACTION OF A CLAY FROM THE COLOMBIAN AMAZON

Abstract

Natural antibacterial clays can inhibit growth of human pathogens; therefore, understanding the antibacterial mode of action may lead to new applications for health. The antibacterial modes of action have shown differences based on mineralogical constraints. Here we investigate a natural clay from the Colombian Amazon (AMZ) known to the Uitoto natives as a healing clay. The physical and chemical properties of the AMZ clay were compared to standard reference materials: smectite (SWy-1) and kaolinite (API #5) that represent the major minerals in AMZ. We tested model Gram-negative (*Escherichia coli* ATCC #25922) and Gram-positive (*Bacillus subtilis* ATCC #6633) bacteria to assess the clay's antibacterial effectiveness against different bacterial types. The chemical and physical changes in the microbes were examined using bioimaging and mass spectrometry of clay digests and aqueous leachates. Results indicate that a single dose of AMZ clay (250 mg/mL) induced a 4–6 order of magnitude reduction in cell viability, unlike the reference clays that did not impact bacterial survival. AMZ clay possesses a relatively high specific surface area (51.23 m^2/g) and much higher total surface area (278.82 m^2 /g) than the reference clays. In aqueous suspensions (50 mg clay/mL water), soluble metals are released and the minerals buffer fluid pH between 4.1 and 4.5. We propose that the clay facilitates chemical interactions detrimental to bacteria by absorbing nutrients (e.g., Mg, P) and potentially supplying metals (e.g., Al) toxic to

bacteria. This study demonstrates that native traditional knowledge can direct scientific studies.

Introduction

Clays and clay minerals have medicinal applications that could be used to respond to environmental and health challenges, such as the re-emergence of infectious diseases, antibiotic resistance, and increasing accumulations of antibiotics in reclaimed water. Clays are < 2μm minerals (estimated spherical diameter), with high relative surface area (10's to 100's m^2/g), variable surface charge, and high ion exchange capacity. In a clay poultice (clay mixed with water), it is the clay that dominates the chemistry of the aqueous phase (pH and Eh); thus, clay minerals control the aqueous speciation in the system and determine the chemical reactions possible (Williams et al., 2009).

Certain natural clays can inhibit growth of human pathogens (Brunet de Courrsou, 2002; Cunningham et al. 2010; Williams et al., 2004, 2008, 2009, 2011; Williams & Haydel, 2010; Morrison et al., 2014). The antibacterial mechanisms of action for natural clays seem to be diverse. In vitro studies indicate that certain metals, soluble at the conditions buffered by the clay, have deleterious effects on bacteria via production of reactive oxygen species (ROS; Cunningham et al., 2010; Morrison et al., 2014). Many antibacterial clays can generate aqueous leachates that are antibacterial as well, indicating a role of soluble species in the process, while others do not. Therefore, we examine the various modes of action of antibacterial clays.

Here, we report on a natural clay from the Colombian Amazon (AMZ) that we found to be bactericidal against model Gram negative and Gram positive bacteria. This clay is used by the Uitoto people, a native culture who live in the Colombian Amazon region, and who ingest it to cure gastrointestinal problems. The clay was collected and studied with permission from a Uitoto traditional authority. The clay deposit formed in a lacustrine environment that existed in the Early to Middle Miocene (Hoorn, 1994). Its potential antibacterial effect was tested *in vitro*, and compared to mineralogical controls. The goal was to determine if the health effect realized by the Uitoto people related to bactericide and if so, was the mode of action a physical (electrostatic) or chemical process. Results suggest that nutrient limitation by the clay and a surplus of metals are the main drivers of the AMZ's bactericide.

Materials and Methods

Antibacterial Clay

The AMZ clay sample was identified as a healing clay by members of the Uitoto tribe. The sample comes from the Eocene-Miocene age, Pebas Formation: this time was marked by continental flooding. The Pebas Formation was deposited in fluvial environments that graded into swamps and lakes (Hoorn & Wesselingh, 2010). The clay size fraction (<2 μm) was separated from bulk clay samples by standard centrifugation methods (Jackson, 1979; Moore & Reynolds, 1997).

Reference Clays

Kaolinite API #5 from Bath, Lamar Pit, South Carolina, was obtained from the American Petroleum Institute reference collection (Molloy & Kerr, 1961). It was chosen as a pure kaolin control in microbiology experiments because of the high percentage of kaolin minerals present in AMZ clay. In addition, Wyoming smectite SWy-1 was obtained from the Source Clay Repository of the Clay Minerals Society, Purdue Indiana (Moll, 2001). It was chosen as a control due to the high content of smectite mineral in AMZ. Previously it was shown not to be bactericidal (Williams et al., 2008) but here it was used for testing surface energy and potential attraction to bacterial cells.

Bacterial Strains

Organisms were purchased from American Type Culture Collection (ATCC). Two species, one representative of Gram-negative bacteria (*Escherichia coli* ATCC 25922) and one representative of Gram-positive bacteria (*Bacillus subtilis* ATCC 6633) common to human digestive tracts were studied.

Leachates

Aqueous leachates of the clay were obtained by mixing 50 mg clay/mL deionized (DI) water in a wrist-action shaker for 24 h, followed by centrifuging at high speed (26,892 × *g* for 1 h, Sorvall RC 5c) to settle particles >0.03 μm. The leachate was decanted, sterilized in the autoclave (20 min, 120 °C), and tested against bacteria. The clay–water equilibration time was set to 24h (Williams et al. 2011). To test the effect of growth media on mineral dissolution, a Luria Broth-leachate was prepared by incubating

(37°C, 24 h) clay with LB (80 mg clay/mL) to mimic the minimum inhibitory concentration (MIC) of the antibacterial experiment. The clays were separated by centrifugation prior to ICP-MS analysis.

Exchange Clay

AMZ clay was shaken with 1N KCl (10 mg clay: 1 mL exchange solution) for 24 h, leaving K-saturated cation exchange sites in the clay (Jackson, 1969). The minerals were separated by centrifugation and washed until chloride was removed, and this sample is referred as AMZ Xc. Exchange solutions analyzed by ICP-MS were prepared using 1M NH_4 acetate instead of KCl, in order to avoid running solutions high in chloride.

Characterization of Clays and its Derivatives

Clay mineralogy was studied by X-Ray diffraction using a Bruker D5000 diffractometer with CuKα radiation. The quantitative mineralogy was performed using methods described in RockJock (Eberl, 2003), a full XRD spectral-matching program for randomly oriented powder analysis, using alumina (10%) as an internal reference mineral. Clay morphology was characterized using Scanning Electron Microscopy (SEM) following the methods presented in Bennett et al. (2006). Samples for SEM were C-coated for charge compensation, and observed under the XL30 FE-SEM using 20 kV acceleration voltage.

To characterize the clay surface, measurements were made of the total specific surface area (TSSA), specific surface area (SSA), and cation exchange capacity (CEC). TSSA was measured following the ethylene glycol monoethyl ether (EGME) method

(Tiller & Smith, 1990). TSSA includes the clay mineral interlayer; it is the maximum area available to interact with water, ions, and polar molecules (Środoń & McCarthy, 2008). The SSA does not include the clay interlayer. SSA was measured based on N_2 adsorption to the exterior mineral surfaces following the Brunauer, Emmett and Teller (BET) method (Dogan et al., 2006). CEC was measured with a spectrophotometric method using cobaltic hexamine chloride (Aran et al., 2008). Measurements of pH and Eh were obtained on 250 mg/mL clay suspensions in deionized (DI) water equilibrated for 24 h, using a Thermo Scientific ORION Dual start meter (Williams et al., 2004). The 24 h equilibration time was chosen to mimic the appropriate time of changing poultices to treat topical infections (Williams, et al., 2004).

Zeta Potential (ζ) and DLVO Theory

A zeta potential analyzer (ZetaPALS; Brookheaven Instruments) was used to measure bacteria and clay mobility. Zeta potential (ζ) was calculated using the Smoluchowski equation, at the leachate electrochemical conditions (pH 4.0-4.6; Ionic strength= 0.01 mM; Eh = 50–70 mV at 25°C). The ζ of clays was determined in a suspension of 1 g clay/L leachate using both the AMZ leachate and the kaolinite API#5 leachate. Bacterial cells were grown in 50 mL to ~10^9 CFU/mL (OD 600nm = 10^9 CFU/mL = 1 g/L), harvested at exponential growth phase, pelleted, rinsed with 0.8% NaCl, and re-suspended in the clay leachate. Values reported represent the average of six independent analyses.

DLVO theory was used to calculate the total interaction energy in a divalent electrolyte as a sphere, which represents the bacterial cell, and a platy particle, which

represents the clay. The total interaction energy is the result of adding the repulsive electrostatic and the van der Waals attractive energies. The repulsive electrostatic energy was calculated following Hogg et al. (1966) and using the ζ values as the surface potentials. Van der Waals attractive energy was calculated using the equation in Gregory (1981) with a Hamaker constant of $6.5X10^{21}$ J for the bacteria cell-water-quartz system (deKerchove & Elimelech, 2005; Redman et al., 2004).

The chemical composition of the clay fraction (< 2μm), leachates, and cation exchange solution were determined using spectroscopic and spectrometric methods. Major elements in AMZ were determined using X-ray fluorescence (XRF). Analyses were conducted at the U.S. Geological Survey (Denver, CO) and at the National University of Colombia in Bogotá. Minor and trace elements in cells, clay separates, cation exchange solution, aqueous and LB leachates, and growth media (LB), were quantified by inductively coupled plasma mass spectrometry (ICP-MS) in a 2 % HNO_3 matrix. A Thermo electron X-series quadrupole (Q-ICP-MS) in the Keck lab at Arizona State University (ASU) was used. The anions in the leachate were determined using ion chromatography (IC) in a DX600 Ion Chromatography system (Dionex). To determine the elemental concentration sourced by the clay in the LB leachate, the elemental composition of LB was subtracted from the LB leachate.

Antibacterial Assays

Bacterial strains were grown overnight in Luria Broth (25 g LB/L) at 37°C on a rotating drum. In vitro antimicrobial susceptibility tests were conducted following procedures described in Williams et al. (2008). Briefly, 400 μl of liquid culture (~10^8

CFU/mL) in exponential growth phase was incubated overnight at 37°C with 100 mg of autoclaved clay (250 mg/mL). The incubated mixture of bacteria and clay in LB suspension was serially diluted and plated on LB agar (25 g/L LB). Colonies were counted and converted to cell density (colony forming units; CFU) (National Committee for Clinical and Laboratory Standards [NCCLS], 2000). Controls for bacterial growth in the absence of clay and in the presence of the kaolinite reference clay (API #5) were included. The antibacterial activity of the AMZ leachate was tested by mixing 1 mL aqueous leachate to 1mL bacteria suspended in LB (10^8 CFU/mL) and plating as described (NCCLS, 2000).

Removing Organics

Water-soluble and volatile organics were removed from the AMZ during size separation and autoclave treatments. To rule out possible action of natural organic compounds present in the clay, additional organic extraction was performed by mixing 1 g of AMZ clay with 5 mL dichloromethane (DCM) and 5 mL methanol (MEOH), hand shaking for 5 min and soaking overnight. The solvents with the extracted organics were separated and the treatment was repeated three times. After treatment with DCM/MEOH, the clay was dried at 60°C overnight, ground, sterilized, and tested in duplicate against *E. coli* at two concentrations (70 and 100mg/mL) following standard serial dilution and plate counting methods (NCCLS, 2000).

Bacteria–Mineral Separation

To study the chemical composition of *E. coli* and clay after the antibacterial assay, a cell-mineral separation procedure was used (Neveu et al., 2014). Bacteria and

clay were incubated following the antibacterial testing protocol described above. More dilute media (5 g LB/L) was used in order to minimize metal precipitation from soluble clay elements. In brief, 5mL of extraction buffer (5 mL phosphate buffer saline (PBS) at pH 7.4; 1 mg Na-pyrophosphate; 0.25 mL Tween detergent) were added to the *E. coli*–clay suspension. The samples were stirred at 720 rpm for 30 min and sonicated in a low energy water bath for 1 min. The samples were transferred on top of a 5 mL layer of Nycodenz density gradient media (Axis-Shield Cat. No. 1002424, density adjusted to 0.8 mg/mL). Final separation was achieved by centrifugation in a swing-arm centrifuge (Eppendorf Benchtop Centrifuge 5804R, 5000 × *g*, 50 min, 4 °C). The sediments (clays > 800 mg) settled at the bottom and the bacteria (<80 mg) floated on top of the Nycodenz layer. The presence of intact bacterial cells was confirmed under the light microscope. The recovered bacterial cells were washed three times with EDTA-oxalate to remove metals adsorbed on the cell membranes (Tovar-Sanchez et al., 2003). A control of *E. coli* grown in LB (5 g/L) without clay was included. All experiments were performed in triplicate.

Element Exchange Between Clay and Bacteria

To test for exchange of elements between clays and bacteria, the chemical composition of each was analyzed using ICP-MS after incubation for 24 h at 37 ˚C. After bacteria–mineral separation, the separated phases (reacted clay and bacteria) were dried in a clean hood and weighed. A control of *E. coli* grown overnight in LB (5 g/L) without clay was included. Samples were prepared in triplicate. Samples were re-suspended in 5 mL concentrated HNO_3 and transferred into Teflon beakers for acid digestion. Bacterial

cells were digested overnight in concentrated HNO_3 and H_2O_2 on hot plate at sub-boiling temperature (130 °C) followed by additional digestion in concentrated HNO_3. Clays were digested in concentrated HF and HNO_3. A second digestion was performed using concentrated HCl at 130 °C. Finally, acids were evaporated, and the samples were diluted in a 2 % HNO_3 matrix for multi-elemental analyses by ICP-MS.

Transmission Electron Microscopy (TEM)

Sample mounts for TEM also required cell separation from the bulk of the clay, but different bacteria–clay separation protocol was followed in order to preserve some of the minerals, allowing observation of the physical interactions between the mineral and bacteria surfaces. For TEM, bacteria were incubated with clay at the minimum inhibitory concentration (MIC = 80 mg clay/mL culture; ~10^8 CFU/mL). A total of 500 µl of the mineral/bacteria suspension was mixed with 1 mL phosphate buffered saline (PBS) solution, 1 mL oxalate basic solution (Tovar-Sanchez et al., 2003), and 10 mg of Na-pyrophosphate. The sample was vortexed at minimum speed for 10 min and sonicated in a bath for 1 min. Minerals plus bacteria were separated by centrifugation (using a Heraous Labofuge 400 centrifuge, 110 g, 3.5 min). The supernatant contained mostly bacteria and this was decanted into a sterile test tube; the procedure was repeated three times to increase the number of bacteria collected. The separated fraction was pelleted and fixed in 2% glutaraldehyde (GA) in PBS for 2 h.

TEM mounts. For the control bacteria, 500 µl of liquid culture (~10^8 CFU/mL) were rinsed three times with PBS, pelleted, and fixed with 1 % GA in PBS. To obtain dense cell aggregates, samples were then pelleted into 0.8 % agarose. Cells were treated

with 1 % OsO_4 for 2 h, washed with DI water, and in-bloc stained with 0.5 % uranyl acetate aqueous overnight at 4 ˚C. Cells were washed, dehydrated in a graded acetone series, and infiltrated with Spurr's epoxy resin (Spurr, 1969) over a 2-day period. Specimen blocks were polymerized at 60˚C for 24 h. Ultrathin (70 nm) sections were obtained with a Leica Ultracut-R microtome and collected on copper slotted grids coated with formvar. Sections were post-stained with 2 % uranyl acetate in 50 % ethanol and Sato's lead citrate (Hanaichi et al., 1986). TEM images were generated with a Philips CM12 TEM operated at 80 kV and digitized with a Gatan model 791 CCD camera. For energy–dispersive X-ray spectra (EDS), the specimen blocks were cut to ultrathin (50 nm) sections, collected on Au-grids, and C coated to avoid charging and to increase stability of the sample.

Results

Characterization of AMZ Clay

The clay fraction (< 2μm) of AMZ contains quartz, a mixture of various clay minerals, and accessory phases as indicated by random powder X-ray diffraction (Figure 3.1). The clay minerals are primarily 1:1 kaolins (44 %) and 2:1 clay minerals (38 %) (Table 3.1).

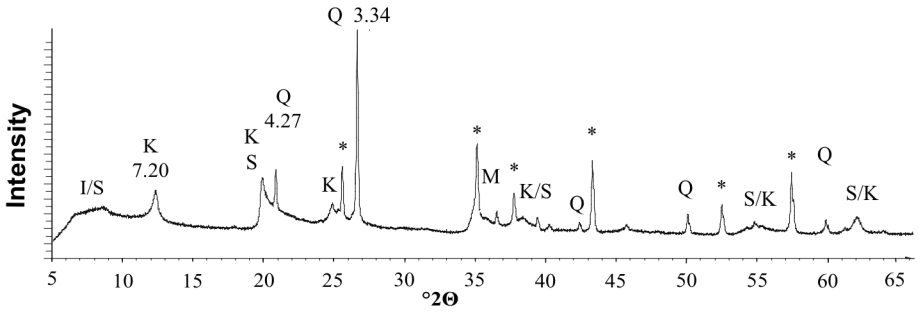

Figure 3.1. Interpreted XRD pattern of the random powder using an alpha-alumina standard (*asterisk*). K, kaolin- group minerals; Q, quartz; S, smectite; M, Muscovite; I+S, superposition of illite and smectite reflections; S+K, superposition of smectite and kaolinite reflections

Table 3.1

Quantitative Mineralogy of the Antibacterial AMZ sample

Mineral	Weight (%)
Quartz	15
Anatase	0.4
Clay minerals	
Halloysite	15.4
Kaolinite	29
Smectite	30
Illite	0.6
Muscovite	6.9
Total clays	81.8
Total	97.9

Full pattern degree of fit: 0.0342. Determined with RockJock (Eberl, 2003).

Chemical analyses by electron microprobe and X-ray fluorescence (Table 3.2) show ~60 % Si, 30 % Al and 4 % total Fe. Major element concentrations are similar to the values reported for the chosen standard clays (Kerr et al., 1951; Van Olphen & Fripiat, 1979), however the AMZ clay contains a variety of minor and trace elements (Table 3.2) that could pose a threat to bacteria if bioavailable.

Table 3.2.

Measurements of Major, Minor and Trace Elements of the Bulk AMZ Clay Compared to the Reference Clays SWy-1 and Kao API#5

Major Elements (wt%)

wt %	Mean	SD	SWy-1	Kao API #5
SiO_2	59.42	4.89	62.9	45.49
Al_2O_3	32.47	1.52	19.6	36.3
Fe_2O_3*	3.72	0.69	3.67	1.3
K_2O	1.89	0.51	0.53	0.52
TiO_2	0.95	0.16	0.09	1.45
Na_2O	0.35	0.16	1.53	0.41
MgO	0.98	0.14	3.05	0.07
P_2O_5	0.05	0.02	0.049	N/D
CaO	0.14	0.08	1.68	0.42
MnO	0.02	0.01	0.06	N/D

Minor and Trace Elements (ppm) in AMZ

Element	Mean ppm	S.D.
V	151.53	5.5
Cr	89.26	3.3
Co	5.56	0.2
Ni	14.00	0.00
Cu	82.00	3.00
Zn	46.00	2.00
As	3.20	0.10
Se	12.00	1.00
Rb	80.00	4.00
Sr	85.00	3.00
Zr	101.00	4.00
Mo	4.20	0.20
Ag	0.11	0.01
Cd	0.09	0.00
Ba	225.00	7.00
Pb	25.63	0.70
U	4.07	0.15

Note. Major elements in AMZ were determined by XRF, reported as oxides.
SD standard deviation, ND not determined.
[a] Data from van Olphen et al. (1979), [b] Data from Kerr (1951).
*Total Fe is reported as Fe_2O_3 wt.%. Minor and trace elements determined with ICP-MS, reported in ppm.

Due to the dominance of both smectite and kaolins in AMZ, the TSSA and CEC of clay lie between these reference clays (Table 3.3). However, AMZ presents a higher specific surface area (SSA= 51.2 m^2/g) compared to the reference clays SWy-1 and kaolinite API #5. The surface texture of the AMZ appears rough, heterogeneous, and morphologically less defined than the reference Kao API #5 (Figure 3.2), which may account for the higher SSA. Because AMZ clay contains almost 50% kaolins, we used the reference kaolinite (API #5) as a non-antibacterial control clay.

Table 3.3

Surface Properties of the AMZ Clay Compared to Two Reference Clays: Kaolinite (Kao API#5) and Smectite (SWy-1)

Property	Smectite SWy-1	Kaolinite API #5	AMZ
Total Surface Area (EGME)	698.81 m^2/g	48.3 m^2/g [a]	278.82 m^2/g
Specific Surface Area (BET)	29.85 m^2/g	17 m^2/g [b]	51.23 m^2/g
Cation Exchange Capacity	76.73 meq/100g	3.00 meq/100g[b]	29.32 meq/100g

[a] Data from Carter et al. (1965); [b] data from Philen, et al. (1971).

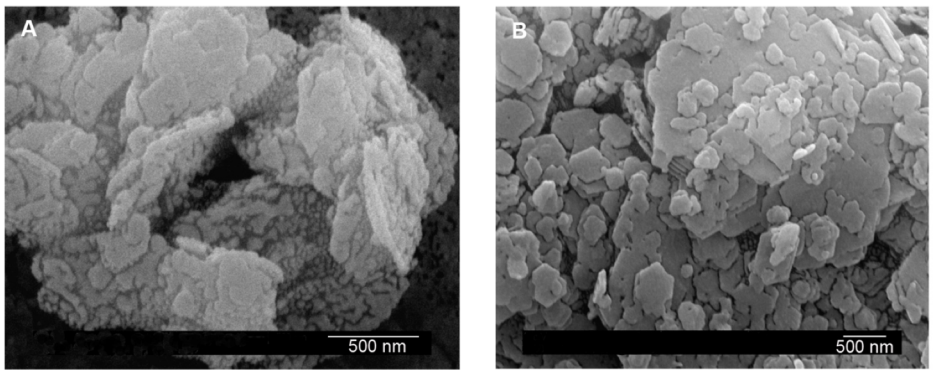

Figure 3.2. SEM images of clay minerals using 80 keV current. A. AMZ clay presents a rough surface with less well-defined crystalline faces compared to reference clays. B. Kaolinite API #5 shows smooth surfaces and well-defined crystals with the typical hexagonal shape of kaolinite

Zeta potential of clays. The mineral surface potential varies as a function of pH. After 24h of equilibration (250 mg/mL), the pH of the water in contact with clay is 4.5 for AMZ and 5.5 for kaolinite API #5. At these pH values, the zeta potential (ζ) of AMZ and Kao API #5 are -14mV and -21mV respectively. The ζ of cation exchanged AMZ (AMZ Xc) did not vary relative to untreated AMZ (Table 3.4).

Table 3.4

Zeta Potential of AMZ and Kaolinite API#5 (Kao#5) at the pH of the Viability Experiments (4.5 and 5.5, Respectively).

Sample	Zeta potential (mV)	pH
B.sub in Kao API#5 leachate	-35	5.5
B.sub in AMZ leachate	-35	4.0
E.coli in Kao API#5 leachate	-35	5.5
E.coli in AMZ leachate	-33	4.1
AMZ in its leachate	-14	4.1
Kao API#5 in its leachate	-21	5.5
AMZ Xc in KNO_3	-15	4.1

Note. The leachate concentration used was 100 mg/mL equilibrated by shaking 24 h at room temperature.

Aqueous leachate, exchange solution and LB leachate. In the aqueous leachate of AMZ, the ion concentration decreases in the order K > Na > Ca > Mg. However, exchanging the clay with KCl removes mostly Ca from the exchange sites, followed by Mg > K > Mn (Table 3.5). Minor elements are slightly more concentrated in the exchange solution than in the aqueous leachate, but their concentration (ppb range) is relatively low compared to the LB leachate (Table 3.5). The LB leachate is enriched in Ca, Mg, and Al, which reach a concentration two orders of magnitude higher than the aqueous leachate and exchange solution. The concentrations of Na, K and P are reported as zero because they are major constituents of LB (Chapter 4, Table 4.3).

Table 3.5

ICP-MS Data of the AMZ Leachate, Cation Exchange Solution and LB Leachate Corrected (LB-LB Leachate)

	AMZ leachate		Exchanged Solution AMZ		LB leachate*
Element	µM	SD	µM	SD	µM
Na	13.03	6.91	14.57	2.03	0
Mg	8.32	0.58	255.7	19.19	1217
Al	0.31	0.20	0.9	0.27	1332
P	0.17	0.08	0.87	0.08	0
K	25.93	1.39	75.32	4.27	0
Ca	10.92	0.78	322.38	26.56	1523
Ti	BDL	N/A	BDL	N/A	BDL
V	0.01	0.00	0.05	0.00	2.28
Cr	BDL	N/A	BDL	N/A	0.16
Mn	0.83	0.09	23.33	1.80	110.42
Fe	0.01	0.00	0.03	0.01	15.62
Co	0.02	0.00	0.13	0.01	3.82
Ni	0.02	0.00	0.06	0.00	3.93
Cu	0.24	0.03	5.45	0.31	52.9
Zn	0.18	0.06	0.56	0.08	41.3
As	BDL	0.00	0.07	0.01	0.12
Se	0.07	0.01	0.73	0.06	N/A
Rb	0.02	0.01	0.65	0.03	0.76
Sr	0.08	0.01	2.42	0.16	13.86
Ba	0.04	0.00	2.59	0.23	11.25
Pb	BDL	0.00	0.08	0.00	0.15
U	BDL	0.00	0.02	0.00	BDL

Note. SD Standard deviation. SD is not reported for LB leachate because it is calculated.

Bacterial Susceptibility to Clay and Leachates

Incubation of AMZ clay (250 mg/mL) with the model bacteria *E. coli* (ATCC 25922) and *B. subtilis* (ATCC 6633) reduced cell viability by 4–6 orders of magnitude relative to kaolinite (Kao API #5) at the same concentration, and to a control population grown without clay (Fig 3.3). The minimum inhibitory concentration (MIC) of AMZ is 80 mg/mL in LB (25 g/L). The aqueous leachate and the LB leachate did not reduce

bacterial viability (data not shown), contrary to clay leachates from previous studies (Williams et al., 2008, 2011).

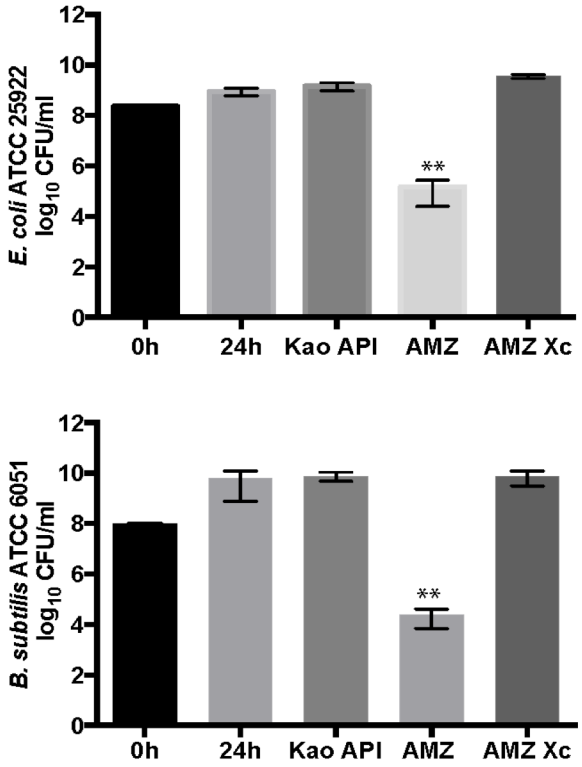

Figure 3.3 Bacterial cell density, from left to right initial conditions (0 h), after 24 h of incubation at 37 ˚C with LB (24 h), and with control clay (Kao API #5), AMZ clay, and cation-exchanged AMZ clay (AMZ Xc). Mineral concentrations were 250 mg/mL bacterial culture. The results reported correspond to 1:1000 dilution for AMZ clay and $1:10^6$ dilution for control clays and cells grown without clay. The reported values represent the average and SD of at least three independent experiments. The difference between the bacteria incubated with AMZ clay and the control (no clay) is statistically significant (**$p < 0.05$, paired t-test)

In order to assess the effect of ions and compounds adsorbed by the clay, we performed two separate treatments prior to viability testing: cation exchange and removal of DCM/MEOH soluble organic compounds. Exchanging the AMZ clay with 1M KCl

(AMZ Xc in Figure 3.3) removed its bactericidal effect. The exchange solution was not tested for bactericide due to the elevated levels of Cl^- that would kill bacteria. Treatment of the AMZ with DCM/MEOH did not impact its antibacterial performance (DCM/MEOH in Figure 3.4). Volatile and water-soluble organics were not of concern since they should have been removed in the consecutive washes performed to separate the < 2 μm fraction or volatilized during sterilization in the autoclave (120 °C for 20 min).

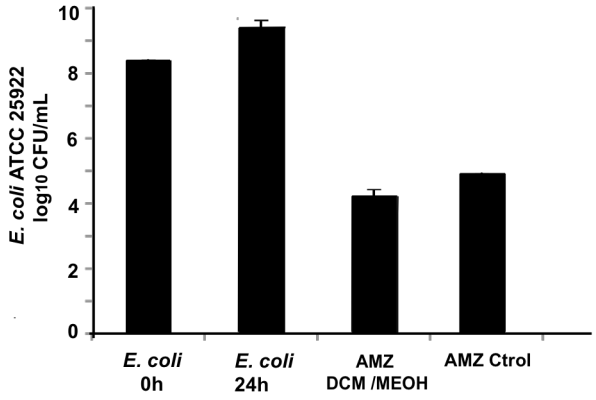

Figure 3.4 Bacterial cell density in CFU/mL before and after 24h of incubation at 37˚C with AMZ clay and AMZ extracted with DCM-MEOH to remove organics. The dose employed was 100mg clay/mL of bacterial culture. The concentration reported corresponds to the 1:1000 dilution. The difference is not significant (p = 1.8) indicating that organic compounds do not cause the bactericidal effect, assuming organic extraction was complete

Bacterial Morphological Changes and Physical Interactions with Clay

Clays and bacterial cells were imaged using TEM to study the physical interaction at the MIC dose of clay minerals (80 mg/mL). Clay minerals appear dark and with well–defined edges; the aluminosilicate composition of these features was confirmed by EDS (Figure 3.6). Kaolinite crystals appear with a characteristic hexagonal shape of the 001 face; the kaolinite in AMZ often had pits on its surface. Some clay minerals seem elongated, needle-like laths with sizes that range from <0.1to 2.0 μm. The control bacteria were imaged in the absence of clay (Figure 3.5 a, b, g, h). The changes in morphology of bacteria reacted with clay were compared to the control.

The morphology and structure of *E. coli* is shown in Figure 3.5a. Both the outer membrane (OM) and the cytoplasmic membrane (CM), along with the periplasmic space are visible. The area identified with letter N shows the genetic material, i.e., DNA coiled in the chromosomes that form the nucleus. The cells were actively dividing, confirming that the cultures were in the exponential growth phase at the time of exposure to clay (Figure 3.5b).

Clays were observed on top of, or in the vicinity of, cells (Figure 3.5, arrow 1), and *E. coli* responses were different for AMZ and Kao API #5, except for the outer membrane structures (Fig 3.5f, arrow 2) produced by both clay samples. Still, exposure to Kao API #5 did not modify the *E. coli* shape or internal texture (Fig 3.5d, f). In contrast, *E. coli* reacted with AMZ showed electron dense (dark) areas in the periplasmic space or in the membrane (Fig 3.5, arrow 3), and distortion of the shape of the cell envelope (Figure 3.5, arrow 4). Using TEM, it was possible to observe clay minerals

engulfed in what appears to be an extracellular polymeric substance of the membrane (Figure 3.6).

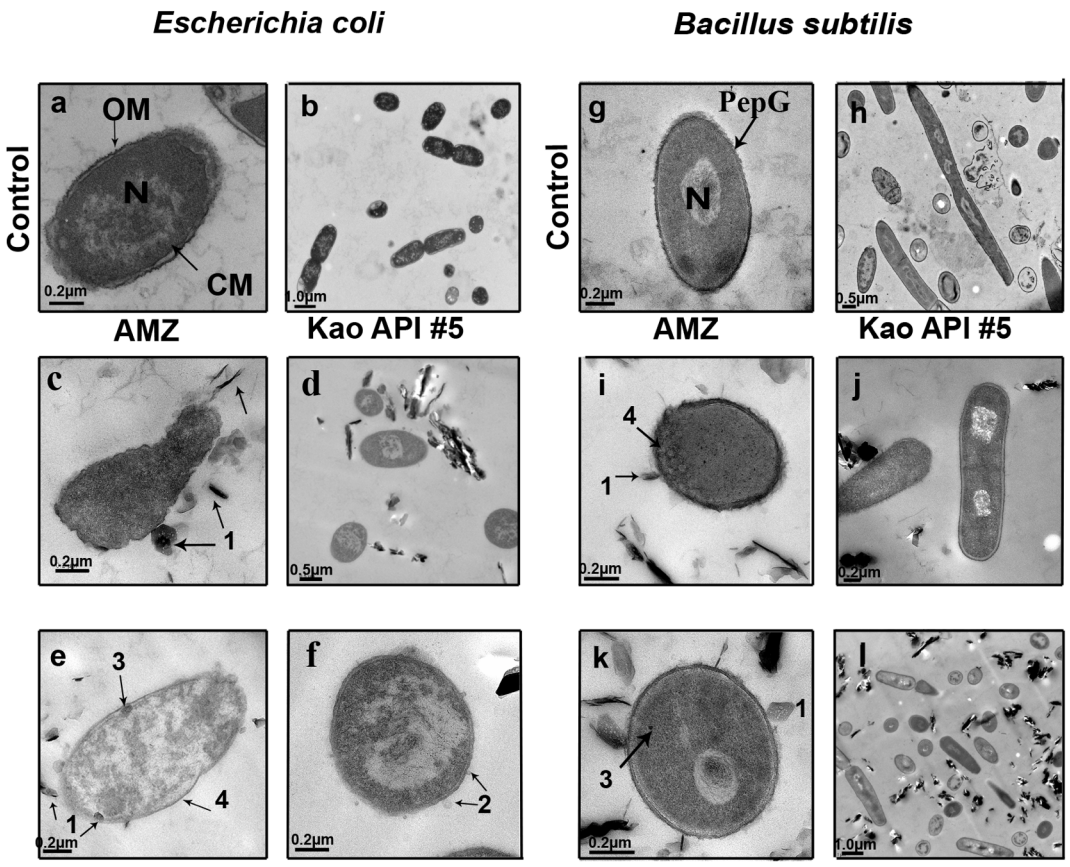

Figure 3.5 E. coli and *B. subtilis* sections imaged by TEM. Photos A, B, G and H are control bacteria not exposed to clays. OM, Outer membrane; CM, Cytoplasmic membrane; N, Nucleotide; PepGly, Peptidoglycan cell-wall. Left columns: *E. coli* reacted with AMZ (C, E) and with kaolinite API #5 (D, F). Right columns: *B. subtilis* reacted with AMZ (I, K) and with kaolinite API #5 (J, L). Numbers in the image indicate: 1) clay minerals, 2) membrane vesicles, 3) intracellular electron dense particles, 4) other textural changes.

Bacillus subtilis showed its typical rod-shaped morphology in the control images (Fig 3.5 g, h). The thicker peptidoglycan (PepG) layer of Gram-positive bacteria and the nucleus are identified in Figure 3.5g. In some instances, clay particles can be observed close to the membrane of *B. subtilis,* but the dark areas that were observed in *E. coli* images are not present in *B. subtilis*. In contrast to *E. coli*, the impacts of AMZ clay on the texture and morphology of *B. subtilis* are subtler. AMZ clay minerals were observed attached to the cell membrane (Figure 3.5, arrow 1). Fewer Kao API #5 minerals were observed interacting with *B. subtilis* cells (Figure 3.5i) compared to textural changes on the membrane that were noticeable on the *B. subtilis* exterior (Figure 3.5, arrow 4) reacted with AMZ. In Fig 3.5j a *B. subtilis* cell is shown in early phases of division after reaction with the kaolin standard, further evidence of the continued viability of the bacteria reacted with Kao API #5.

Electrostatic Interactions of Clays and Microbes

Both bacterial cells and clay minerals have a net negative surface charge (Table 3.4) that determines interaction energies. The zeta potential of *E. coli* and *B. subtilis* measured in the leachates of the two clays is similar, showing an average of –34 mV. There are a 20 mV difference between AMZ clay and the bacteria and a 15 mV difference between the Kao API#5 and bacteria, still both surfaces are negatively charged.

Table 3.6

Energy Barriers between Bacteria and Clay, Secondary Minimum in which Weak Attraction is Possible at The Separation Distance Calculated by DLVO Theory

	Energy barrier (kT)	Secondary minimum depth (kT)	Secondary minimum separation (nm)
B.sub–Kao API#5	469.46	-0.77	38
B.sub–AMZ	360.87	-1.10	36.5
E.coli-–Kao API#5	477.49	-0.72	38.5
E.coli–AMZ	216.22	-0.62	36

Using the ζ as a proxy for surface charge, the energy barriers for irreversible attachment were calculated by DLVO theory (Israelachvili, 2007). Energy barriers are lower between AMZ and bacteria, than Kao API #5 and bacteria (Table 3.6; Figure 3.7) but there is an apparent repulsion between surfaces at short distances. The negative energy barriers present at the secondary minimum (i.e. reversible particle attraction) could occur at separation distances in the range of 35–38 nm (Table 3.6; inset of Figure 3.7).

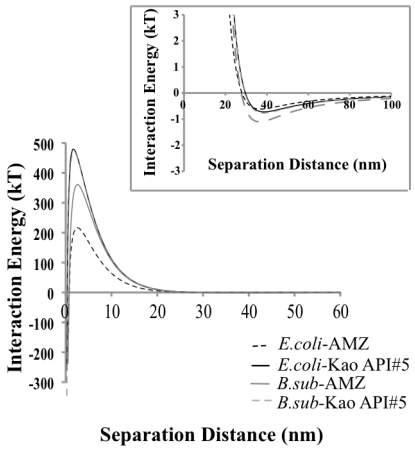

Figure 3.6 Calculated DLVO profiles for the two model bacterial strains and clay minerals. Electrostatic interaction energies were calculated using the zeta potential (Table 3.4).

Chemical Interaction of Clay and *E. coli*

To study the chemical interactions between clay and bacteria, we compared the elemental composition of *E. coli* and the clays after reaction (incubation at 37 ˚C for 24 h) relative to control bacteria grown without clay. Hereafter, we refer to the *E. coli* that was incubated with AMZ clay as '*E. coli* reacted with AMZ' or for experiments with Kao API#5 '*E. coli* reacted with Kao', and to the clay that was mixed with bacteria as the "reacted clay". *E. coli* incubated without clay is the 'control *E. coli'*.

E. coli reacted with AMZ showed a decrease in the Mg, P, and K concentrations relative to the control *E. coli* (Table 3.7). The concentration of Al, Se, and V is notably high (two orders of magnitude greater than the control) in *E. coli* reacted with AMZ, and the concentration of metals including Fe, Co, Ni, Cu, Cd, Pb and Cs also increased. However, no change (within error) was observed for Ca, Rb, Mo, and Mn, relative to the control (Table 3.7). Na is excluded from the analysis because it is a major component of reagents used, and not a true indicator of exchange between clay and bacteria. The *E. coli* reacted with Kao consistently showed a ~one-fold increase in the concentrations of elements except for V and Al that increased 2 and 1 orders of magnitude, respectively. Realizing that the separation of clay from bacteria is not perfect, we estimated the contribution of clay to the bacterial population considering elements found only in the clay (e.g., Ba, Pb, W) to estimate a percentage of clay-related elements to subtract (Table 3.7).

Table 3.7 *Elemental Composition of* E. coli *Cells Before and After Reaction With Clays*

	E. coli EDTA washed		*E. coli reacted with AMZ*		Corrected E. coli for 3.2% AMZ		Corrected E. coli for 4.5% AMZ		*E.coli reacted with Kao API#5*		Corrected E. coli reacted with Kao API#5 (1%)	
	Average (ppm)	S.D.	Average (ppm)	S.D.	Average (ppm)	S.D.	Average (ppm)	S.D.	Average (ppm)	S.D.	Average (ppm)	S.D.
Mg	562	43	264	63	163	59	110	59	588	127	580	98
Al	34	35	7166	986	2112	1028	0	1045	979	251	-676	517
P	8826	507	4663	1495	4620	1500	4601	1502	11705	2511	11701	2055
K	1965	31	741	116	377	114	225	112	5135	1164	5132	895
Ca	15	4	73	4	35	5	19	5	61	5	52	2
Ti	13	1	24	5	-96	7	-146	8	9	2	-119	9
V	0.05	0.0	7.54	1.2	2.31	1.3	0.12	1.3	1.72	0.2	0.02	0.1
Cr	0.49	0.1	5.57	0.6	2.30	0.7	0.93	0.7	0.96	0.2	-0.32	0.0
Mn	2.54	0.2	8.38	2.6	4.18	2.1	2.92	2.0	3.30	0.8	3.23	0.6
Fe	82	13	1231	115	464	136	144	145	114	25	91	20
Co	0.30	0.1	1.29	0.3	1.13	0.3	1.07	0.3	0.39	0.0	0.30	0.0
Ni	0.86	0.1	2.39	0.1	1.94	0.1	1.73	0.1	1.18	0.2	0.81	0.1
Cu	3.88	0.1	24.87	8.7	17.50	5.5	16.31	5.5	3.71	1.0	3.49	0.8
Zn	14.52	0.6	30.84	4.2	25.73	1.3	24.57	1.4	15.84	2.8	15.52	2.4
As	0.08	0.0	0.54	0.1	0.35	0.1	0.30	0.1	0.42	0.1	0.35	0.0
Se	0.09	0.0	5.27	1.4	4.37	1.4	4.00	1.4	1.19	0.3	0.38	0.1
Rb	0.69	0.1	3.54	0.9	0.86	0.8	-0.44	0.8	1.64	0.5	1.63	0.4
Sr	0.16	0.0	3.08	0.5	0.44	0.5	-0.66	0.4	1.77	0.3	0.78	0.2
Zr	0.18	0.1	3.51	0.7	0.44	0.6	-0.84	0.5	0.51	0.1	-0.07	0.1
Mo	0.97	0.1	0.85	0.1	0.71	0.1	0.65	0.1	2.12	0.6	2.11	0.6
Cd	0.06	0.0	0.25	0.1	0.18	0.0	0.17	0.0	0.46	0.5	0.46	0.2
Cs	0.01	0.0	0.67	0.4	0.33	0.2	0.10	0.2	0.01	0.0	0.01	0.0
Ba	0.45	0.1	6.93	1.7	-0.24	0.2	-3.63	0.2	1.28	0.2	-0.06	0.1
Hf	0.005	0.0006	0.12	0.06	0.05	0.02	0.001	0.02	0.02	0.002	-0.004	0.003
Pb	0.38	0.1	2.46	0.6	1.48	0.8	1.04	0.7	0.60	0.2	0.10	0.1

Discussion

AMZ clay reduces the viability of the Gram-negative *E. coli* and the Gram-positive *B. subtilis,* despite their different cell wall structures. Because the leachates of the clay (both aqueous and LB) did not reduced bacterial viability, it is clear that the clay itself promotes bactericide. To cause viability loss, the AMZ clay must affect or circumvent the cell wall, either by physical or chemical means. We hypothesize that the AMZ could be (1) releasing soluble species that react with bacterial membranes, (2) depriving bacteria of essential nutrients by absorption to the clay surface, or (3) physically attaching to the bacteria in way that could mechanically or electrostatically interfere with their metabolism.

Physical Interactions of Clay and Bacteria

AMZ has a greater specific surface area (external surface) compared to the clay reference materials, smectite and kaolinite (SSA, Table 3.3), and as previously noted, likely relates to the AMZ surface roughness (Figure 3.2). Not only a rough surface enhances the area for reactions but its heterogeneous morphology, composition, and charge distribution might influence physical interactions between AMZ and bacteria.

Electrostatic interactions occur between two charged particles, but both clay and bacteria have predominantly negatively charged surfaces. In the clay structure, the cation substitutions (e.g., Al^{3+} for Si^{4+}) give the mineral a net negative basal surface charge. However, clay minerals can have a positive charge on defects and edges where there are broken bonds (Moore & Reynolds, 1997). The mixed assemblage of minerals in the AMZ clay fraction act to buffer the water pH consistently near 4– 4.5, at a mineral/water

ratio of 50 mg/mL. Localized positive charges could attract to negative charges located on bacterial cell surfaces. In bacteria, the surface charge is also variable and controlled by functional groups that protonate or deprotonate as a function of water pH (Jiang et al., 2004). The ζ of the particles suspended in the leachate is a proxy for surface charge under the aqueous chemical conditions produced by the clay (pH 4.4 for AMZ clay & 5.5 for Kao API #5).

Measurement of zeta potential requires a background electrolyte for charge transfer. The aqueous leachate of AMZ reproduces the complex chemical environment that the particles experience in the clay-bacteria suspension, so we used the natural leachate as the electrolyte, which can complicate the DLVO results (Israelachvili, 2007). Classic DLVO theory predicted that in the case of our experiment, high-energy barriers would act against particle attachment (see Figure 7 and Table 6). However, discrepancies between theory and observations have been reported in studies in which surface roughness is considered (Shellenberger & Logan, 2002). In the presence of rough surfaces, the repulsive energy between particles is reduced (Bhattacharjee et al., 1998) and favorable sites for attraction multiply. Similarly, in this study predictions of substantial energy barriers are at odds with TEM observations of particles that remain attached to cells in spite of cell separation treatments (basic oxalate reagent). Furthermore, adsorption of bacterial envelopes of *E. coli* and *B. subtilis* has been documented for smectite SWy-1 and kaolinite KGa (Walker et al., 1989). While DLVO theory does not accurately portray the complexity of our system, there is clearly not a

large attraction of the AMZ clay to cell surfaces such that suffocation or blockage of efflux pathways is occurring as discussed below.

Morphological Changes in Bacterial Cells

Physical contact seems to occur between the AMZ nanometric clay (<100 nm) and *E. coli* membranes, as shown by TEM imaging (Figure 3.5, 3.6). The images shown in Figure 3.5 are representative of hundreds of bacterial cells studied and meant only as examples of the clay-bacterial interaction. Nanoparticles can compromise the integrity of the cell envelope by producing reactive oxygen species (ROS) that cause lipid peroxidation (Nel, 2006; Neal, 2008; Kibanova et al., 2009); therefore, it is possible that AMZ nanoparticles acted on the bacteria in this way in our experiments. The AMZ nanoparticle shown in Figure 3.6 appears to be wrapped by a thin layer of an extracellular polymeric substance (EPS); this could be a strategy to immobilize threatening particles or a consequence of electrostatic attraction. EPS protects the cell from environmental stresses (Geesey et al., 1988; Harrison et al., 2007) and may form in the presence of toxic minerals as a shielding mechanism (Bruins et al., 2000).

Both EPS and the membranes of Gram-negative and Gram-positive organisms contain weakly acidic functional groups (carboxyl, phosphoryl, amines, and hydroxyl) that attract H^+ under low-pH conditions (Cao et al., 2011). Furthermore, H^+ can displace the structural Ca^{2+} and Mg^{2+} in the membrane, altering its stability (Borrok et al., 2005), and increasing the number of acid functional groups available to react with clay or its derivatives (Bevedrige & Koval, 1981). The EPS and the membrane can interact with clay through these functional groups (Beveridge & Murray, 1980; Fein et al.,1997;

Omoike & Chorover, 2004). Alternatively, amine groups on the bacterial membrane can bind to hydroxylated mineral surfaces (Omoike & Chorover, 2004) and carboxyl groups can bind to metals (Gilbert et al., 2005). The functional groups in the peptidoglycan layer of *B. subtilis* can also precipitate metals (Boyanov et al., 2003; Fein,1997; Walker et al., 1989). The metal precipitation is hard to interpret in TEM sections that have been post-stained. However, the photograph shown in Fig 3.5k, arrow 3 appears to contain electron dense precipitates after reaction with AMZ.

The morphological response of *B. subtilis* to the antibacterial clay AMZ is not as apparent as the *E. coli* response. Clay particles can be seen close to or around *B. subtilis* (Fig 3.5i-l), most of them showing an edge on contact. This arrangement has been interpreted as positive polynuclear aluminum complexes in the clay edge binding to negative carboxylate or phosphate groups in the cell wall (Walker et al., 1989). In the same study, the contact of a clay basal surface and a membrane was explained as a cation-bridge facilitated by a metal. Our data are statistically insufficient to determine which type of mineral-microbe contact dominates but both are observed.

Chemical Interaction of Clay and Bacteria

Clays can be a source of soluble metal species able to affect bacterial viability upon reactions with bacteria (Williams & Haydel, 2010; Morrison et al., 2014). However, the aqueous leachate of AMZ contains low levels of soluble metals and is not antibacterial. Exchanging the clay with KCl (K-saturation) eliminated its bactericidal effect (Figure 3.1). While it is possible that the exchange treatment removed a toxic element that was not highly concentrated in the leachate, it also neutralized the surface

charge of the clay and raised the fluid pH to 5.7. If pH-variable charges or metal speciation were part of the bactericide, then neutralization of surfaces caused by the exchange could hinder the AMZ bactericide.

Clays are known to absorb organic molecules, many of which can be toxic such as pesticides. The likelihood of a water-soluble organic contaminant is small since AMZ clay was sonified and rinsed multiple (5–6) times in distilled-deionized water in the process of separating the < 2 μm fraction from the bulk sample. Many volatile organics would also be lost in the autoclaving process performed to sterilize the clay (20 psi, 126 ˚C for 20 min). Organics soluble in DCM and methanol were extracted from the clay with no change in its bactericidal effect (Figure 3.2). Therefore, we infer that the antibacterial effect is not due to organic contaminants.

The differences observed between the chemical composition of *E. coli* reacted with AMZ and the control *E. coli* suggest two possible modes of antibacterial action: metal toxicity and/or bacterial starvation (Table 3.7). These two modes are interpreted from the ICP-MS data that were corrected to account for clay contamination in the separated cells. To approximate the amount of clay contaminating the sample, we first assumed that all of the Al (7,166 ppm) in the *E. coli* reacted with AMZ was contributed by clay contaminants. Using the total Al content of the AMZ (157,942 ppm) we estimate ~4.5% clay contribution (Table 3.7); therefore, all other elements in the AMZ reacted *E. coli* were adjusted by subtracting 4.5% of the clay concentration for that element. Following the same rationale, the *E. coli* reacted with Kao API#5 was corrected for a 1 % contribution from the clay. The separation of kaolinite and *E. coli* was more effective

than AMZ separation perhaps due to the weaker attraction between kaolinite and bacteria than smectite and bacteria (Walker et al., 1989).

Correcting on the basis of Al, the results suggest that the viability loss of *E. coli* reacted with AMZ may be linked to the clay robbing nutrients from the bacteria, because there is no notable contribution of toxic metals to the bacterial population. Table 3.7 shows decreased Mg, P, and K in the *E. coli* reacted with AMZ compared to the controls. Potassium cannot be used as an indicator for mode of action due to the separation protocol employed, but Mg and P concentrations are reliable (Neveu et al., 2014). The high SSA and CEC of AMZ (Table 3.3) could attract these nutrients away from the bacteria, especially if the acidic conditions allow H^+ to outcompete these elements for cell wall sites (Borrok et al., 2005). The smectite content of AMZ may be responsible for most of the cation absorption, but the depletion anionic suggests either adsorption by other minerals (e.g., halloysite) or precipitation. This result differs from the kaolinite control experiment that shows essentially no change in P after reaction with *E. coli* (Table 3.7) and eliminates kaolinite as the absorbing mineral. Therefore, the AMZ clay appears to prevent Mg and P from being used by the *E. coli*.

Metals with elevated concentrations in AMZ reacted *E. coli* after correction based on Ba content are assumed to have been derived from the clay, since the growth media (LB 5 g/L) mainly contributes Na (Chapter 4, Table 4.3). EQ3 software (Wolery, 1992) was used to determine the chemical speciation of the metals in LB leachate at pH 4.5 and Eh 360mV. In the LB leachate, the Al speciation is 84 % Al^{3+}, 15 % $Al(OH)_2^+$ and 1 % AlO^+. Furthermore, the increase of Al in the LB leachate compared to the exchange

solution and aqueous leachate (Table 3.5) indicates that clay minerals are partially dissolving.

Al^{3+} forms complexes with phosphate ligands found both in bacteria and minerals (MacDonald & Martin, 1988). This chemical affinity provides an avenue for membrane damage as Al^{+3} alters lipid-protein interactions (Garcidueñas & Cervantes, 1995), interferes with the membrane electrical potential, and inhibits membrane transport proteins (Xu et al., 2012). In the membrane, Al^{+3} can substitute for Ca^{+2} and Mg^{+2}, the cations responsible for stabilizing the membrane by binding LPS (Hancock, 1984; Borrok et al., 2005). At acidic conditions buffered by the AMZ clay (pH 4.5), *E. coli* susceptibility to metal attack increases due to the displacement of the structural cations by H^{+}. Thus, Al^{3+} can compromise the integrity of the membrane, potentially causing leakage of cytoplasm (Beveridge & Koval, 1981; Garcidueñas & Cervantes, 1995; Williams, 1999). Based on TEM, ICP-MS, and EDS data we deduce that the AMZ clay attack on bacteria includes membrane damage by excess Al^{+3}.

The uptake of Se increased two orders of magnitude in AMZ reacted *E. coli*, reaching 4 ppm; however, this concentration is not likely important for bactericide. At Eh conditions of 360mV and pH 4.5, Se exists in the V valence state ($HSeO_3^-$ and SeO_3^-). However, *E. coli* can grow in SeO_3^- concentrations as high as 1600 ppm and if Se enters the cell it is reduced to Se^0 or incorporated into proteins (Turner et al., 1998).

Trace concentrations of transition metals are essential for cell functioning (Wackett et al., 2004) but elevated doses catalyze cytotoxic reactions (Nies, 1999; Finney and O'Halloran, 2003). For redox-active species, metal toxicity proceeds via production

of ROS (Imlay, 1988; Kimura & Nishioka, 1997; Schoonen et al., 2006; Stohs & Bagchi, 1995). Fe, Cu, Cr, V and Co all undergo redox-cycling reactions that could contribute to the loss of bacterial viability. For example, V uptake increased from 0.08 to 1.8 ppm in *E. coli* reacted with AMZ compared to control. V in the form of vanadate is structurally similar to phosphates and has been shown to inhibit ATPases, the enzymes that catalyze the dephosphorylation of ATP (Pezza et al., 2002). Therefore, we cannot eliminate small amounts of several transition metals, even below their MIC levels, as playing a role in the antibacterial effect of AMZ. However, the more obvious antibacterial mode of action for the AMZ clay appears to be related to the depletion of nutrients by the clays.

Conclusion

The physical and chemical data presented here show that the model bacterial populations were diminished in the presence of the AMZ clay. There is only a weak physical attraction between the clay and bacterial populations studied, which points to a chemical bactericidal effect. The chemical data suggests two main factors that reduce bacterial viability in both Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) models: 1) the uptake or adsorption of nutrients (Mg, P) by the clay, preventing normal metabolic functions and 2), an overall increase in soluble metals, primarily Al^{3+}, derived from dissolution of AMZ clay components. Furthermore, the cell membrane could have been compromised by the low pH buffered by the clay, and the impaired ability of *E. coli* to maintain the structural stability of the outer membrane due to Mg^{2+} loss. The availability of transition metal ions may further challenge the bacteria by oxidative damage to the membrane or interfering with metabolism by altering enzyme specificity.

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Ethical standard

The results of this study were communicated and explained to the Uitoto traditional authority from which the authors received the sample with permission to study it. No monetary benefit has been generated, and the clay is Uitoto's property. This study shows that certain cultural-based practices have a scientific basis.

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CHAPTER 4

THE ANTIBACTERIAL ACTIVITY OF ALUMINUM IN A CLAY FROM THE COLOMBIAN AMAZON

Abstract

Overuse of antibiotics and the consequent rapid adaptation of bacteria have produced antibiotic-resistant strains of bacteria and polluted both water and soil. The long-term costs of this pollution remain unknown. The pressing problem of antibiotic overuse compels us to seek natural alternatives to pharmaceutical antibiotics. One such alternative is natural antibacterial clays. Certain clays can be antibacterial due to their surface characteristics, mineralogy, and chemical composition, but variations in these properties can result in different antibacterial mechanisms. Here, I describe an antibacterial clay from the Colombian Amazon (AMZ) that primarily consists of kaolin, with an antibacterial mode of action linked to the clay major elements.

Methods from microbiology and geochemistry were combined to evaluate the mineral-microbe interactions that cause antibacterial effectiveness. The surface and chemical characteristics of the AMZ clay were studied as well as the elemental distribution in *E. coli*, before and after incubation with the AMZ clay. The effect of nutrient availability (i.e., P) and acidity on *E. coli* survival was investigated. The concentration of hydrogen peroxide (H_2O_2) produced by the clay was measured in order to assess oxidative damage.

E. coli that was treated with AMZ accumulated Al and lost P relative to control *E. coli* that was not treated with clay. The P–depleted *E. coli* population could not be revived by supplementing the media with P. Low H_2O_2 concentrations produced by the AMZ clay (< 1μM) do not support external oxidative damage as the main antibacterial mechanism. However, Al concentrations derived from the clay exceeded the minimum inhibitory concentration (MIC) determined at the acidic pH buffered by the clay, suggesting Al toxicity. Ion imaging by secondary ion mass spectrometry showed that Al was elevated in the cell membrane relative to intracellular levels, and both were higher than the control *E. coli*. The Fe concentration was below the MIC at acidic pH, however ion images showed increased amounts of intracellular Fe in AMZ treated *E. coli.* Aluminum binding to phospholipids in bacterial membranes has been shown to increase permeability and can thereby, enhance intracellular transport of toxic transition metals. These findings highlight importance of Al for amplifying the toxicity of transition metals.

Introduction

The misuse and overuse of antibiotics threatens both environmental and human health by polluting water and soils and stimulating the evolution of superbugs, i.e., antibiotic resistant bacteria (Baquero et al., 2008; Lindsay & Holden, 2006; Martinez, 2009). Antibacterial clays may be an alternative to traditional antibiotics. After use, they can be sterilized and disposed of safely, without polluting the environment. Furthermore, the availability of these common minerals in developing countries, or remote places that lack health services and facilities, could make antibacterial clays a valuable treatment for

the primary care of skin infections. However, to use antibacterial clays safely and effectively, we need to understand how they affect pathogenic bacteria.

The antibacterial activity of certain clays is linked to their chemistry and to the mineral properties that control the pH and the oxidation state of the water (Williams et al., 2008; Morrison et al., 2016). The presence of transition metal ions in the clay, e.g., Fe^{2+}, is an essential component of some antibacterial clay (Williams et al., 2011; Morrison et al., 2014, Morrison et al., 2016). Aqueous Fe^{2+} produces reactive oxygen species (ROS) that can have deleterious effects on cell components such as membranes and DNA (Schoonen et al., 2006). In the presence of Al, Fe toxicity may be enhanced because Al rearranges the membrane structure in ways that potentiate membrane oxidation (Gutteridge et al., 1985; Zatta et al., 2002). More recently, Morrison et al. (2016) showed that one antibacterial clay related to hydrothermal alteration of a volcanic deposit, destroys bacteria via Fe and Al synergy

Most antibacterial clays identified to date are of hydrothermal origin and have both expandable clay minerals (smectite group) and Fe-rich phases (Williams et al., 2011). Aqueous leachates of these antibacterial clays are also antibacterial (Williams et al., 2008; Haydel et al., 2008, Cunningham et al., 2010). This study investigates an antibacterial clay found in the Colombian Amazon (AMZ) that is of sedimentary origin, mainly composed of non-expandable clay minerals (kaolins), and does not have Fe-rich minerals. Notably, the aqueous leachate of the AMZ clay is not antibacterial (Londoño & Williams, 2016), therefore this clay appeared to kill the model bacteria tested (*E. coli* ATCC 25922) via a different mechanism than did previously studied antibacterial clays.

The curative properties of the AMZ clay were first recognized by the Uitoto native people of the Colombian Amazon, who ingest it to treat common digestive problems, like heartburn. Because there is no understanding of the antibacterial action of this clay, Western scientific methods were used to evaluate its potential antibacterial properties.

The aims of this study were to: 1) determine if bactericidal activity is linked to metal ions as indicated for other antibacterial clays, and 2) determine if the observed nutrient depletion (Londoño and Williams, 2016) contributes to the antibacterial activity. The results of this study help expand our knowledge of the antibacterial mechanism for different minerals. This study also scientifically supports the ancient cultural practice of using the AMZ clay for health benefits.

Methods

Clay and its Derivatives

The AMZ clay was first identified as a healing clay by members of the Uitoto tribe, from the Colombian Amazon; they granted permission for this research and kindly donated the samples. The clay size fraction (< 2 μm) was separated from the bulk clay by standard centrifugation methods (Jackson, 1979; Moore & Reynolds, 1997). A reference kaolinite (KGa-1) and a reference smectite (SWy-1), from the Source Clay Repository of the Clay Minerals Society (Purdue, IN), were chosen as control (e.g., non-antibacterial minerals).

Clay mineralogy was determined by X-Ray diffraction with a Bruker D8 diffractometer with CuKα radiation using a Scintillation detector. The samples were prepared using α-alumina as an internal reference (Eberl, 2003) and mounted in a capillary tube to increase random orientation. The quantitative mineralogy analysis was performed in RockJock, a full XRD spectral-matching program for randomly oriented powder analysis (Eberl, 2003).

Kaolinite disorder in the AMZ clay. The internal disorder of a mineral can increase its solubility by increasing the number of reactive sites (Holdren & Speyer, 1985; Lasaga & Blum, 1986). The Hinckley index (HI) is an empirical method for assessing the amount of disorder in the crystalline structure of clay minerals (Hinckley, 1962). It is calculated from the X-ray diffraction pattern of the AMZ without background correction. The sum of peak heights for the $1\bar{1}0$ and $1\bar{1}1$ crystallographic planes, minus the inter-peak background, is divided by the height of the reflection $1\bar{1}1$ measured from the general background (Figure 4.1; Plançon et al., 1988). A lower peak intensity, (lower HI) indicates increased disorder in the crystal periodicity (Brindley et al., 1986; Plançon et al., 1988).

To characterize the AMZ mineral surface properties, measurements were made of total specific surface area (TSSA), which includes the clay mineral interlayer (Srodon & McCarthy, 2008), specific surface area (SSA), which does not include measurement of the interlayer surfaces (Dogan et al., 2006), and cation exchange capacity (CEC: Aran et al., 2008). The TSSA was determined following the ethylene glycol monoethyl ether (EGME) method (Tiller & Smith, 1990). The TSSA is the maximum area available to

interact with water, ions, and polar molecules (Srodon & McCarthy, 2008). The SSA was measured based on N_2 adsorption to the exterior mineral surfaces following the Brunauer, Emmett, & Teller (BET) method (Dogan et al., 2006).

The CEC was measured using hexamine cobalt (III) chloride solution (Ciesielski et al., 1997), applying the modified method described in Aran et al. (2008). To determine the CEC, 0.7 g dry weight of AMZ was mixed with 25 mL of a 0.04 N hexamine cobalt (III) chloride solution, sonified for 2 min, and shaken for 1 h in a wrist-action shaker. Clays were separated by centrifugation (9000 rpm, 45 min) and 5 mL of the supernatant were transferred into plastic test tubes. The CEC is estimated by measuring the $Co(NH_3)_6^{3+}$ remaining in solution after exchange using a UV/VIS spectrophotometer; the colored solution absorbs at 472 nm. The absorbance of the 0.5 M hexamine cobalt solution was compared to the supernatant to determine the amount absorbed. The assay was performed in duplicate. CEC in meq/100g was calculated using the equation in Aran et al, (2008; see appendix D for details).

Clays were imaged using Scanning Electron Microscopy (SEM) following the methods presented in Bennett et al. (2006). Samples for SEM were carbon-coated for charge compensation, and observed on a Philips XL30 FE-SEM, using 20 kV acceleration voltage, and 10 mm working distance.

Geomicrobiology Methods

Viability testing. To investigate the antibacterial activity of AMZ clay a model Gram-negative bacteria *Escherichia coli* was chosen. *E. coli* is a facultative anaerobe that commonly lives in the gastrointestinal tracts of warm-blooded organisms. The *E. coli* (ATCC 25922) was purchased from American Type Culture Collection (ATCC) and cultured in Lysogeny broth (LB).

Bacteria were incubated overnight at 37 °C and sub-cultured in LB broth (5 g/L)[3], under gentle agitation in an orbital shaker. In vitro antimicrobial susceptibility tests were conducted following procedures modified from Williams et al. (2008). Briefly, 1 mL of liquid culture (~10^8 Colony Forming Units per mL, CFU/mL) in exponential growth phase was incubated overnight and mixed with amounts of autoclaved clay that varied according to the goal of each experiment. The incubated clay-bacteria suspension was then serially diluted, plated in LB agar, incubated another 24 h, and viable colonies were manually counted (Clinical Laboratory Standards Institute [CLSI], 2015). Susceptibility to the clay was assessed by comparing bacterial growth in the samples incubated with clay vs. the samples incubated without clay (control *E. coli*). All experiments were performed in triplicate.

[3]In this chapter, the great majority of the experiments were performed in LB broth prepared at a concentration of 5 g LB/mL. It can be assumed that this is the LB concentration used, unless otherwise stated.

Bacteria-mineral separation. To study the chemical interactions between the AMZ clay and the *E. coli*, the chemical composition of the AMZ was measured before and after incubation with *E. coli*. After incubation with *E. coli*, the clay was separated from the bacteria and both populations (clay and bacteria) were analyzed using ICP-MS and compared to unreacted clay and bacteria. Samples were dried in a clean hood and weighed. Sample digestion for ICP-MS was achieved using EPA method 3050B (EPA, 1996). Briefly, clays were acid digested on a hot plate (130 °C) by repeated additions of 12 M HCl and 16 M HNO_3. Enough acid was added to complete dissolution. The acid was evaporated and the samples were diluted in a 2 % HNO_3 solution for multi-element analyses by ICP-MS. Samples were analyzed via Quadrupole Inductively Coupled Plasma Mass Spectrometer (Thermo Icap Q-ICP-MS) using an in-house multi-element ICP calibration standard, prepared gravimetrically from high-purity ICP-MS calibration standards, and a 5-element internal standard (e.g., Sc, Ge, Y, In, Bi). Running a standard after every six samples during a run assessed instrument performance. Precision was assessed via repeated analysis of the standard through the run; precison ranged from 2-9 % depending on the element being analyzed.

To study the exchangeable cations in the clay, the AMZ was saturated with 1 M NaCl at a proportion of 100 mg clay/ mL NaCl solution (Hendershot et al., 1979; Jackson, 1969) Particles > 0.03 μm were settled by centrifuging at high speed (9,000 rpm for 1h, Sorvall RC 5c). The supernatants of the leachate and exchange solution were transferred into acid-washed tubes and prepared for ICP-MS analysis. Realizing that the LB growth media might exchange elements with the clay and change the aqueous

speciation, the LB broth was incubated at 37 °C for 24 h, with autoclaved clay and the chemical composition of the solution was analyzed via ICP-MS to determine the solution composition for speciation modeling.

ICP-MS of liquids. The chemical composition of the AMZ cation exchange solution and the solution extracted from AMZ incubated in LB were quantified by inductively coupled plasma mass spectrometry (ICP-MS) in a 2% HNO_3 matrix to stabilize trace metals in solution (EPA method 200.8, Brockhoff et al., 1999). The samples were analyzed in a Thermo electron X-series quadrupole (Q-ICP-MS) at Arizona State University (ASU) as described above. Precision ranged from 1-2 % depending on the element being analyzed. Elemental concentrations were determined for Mg, Al, P, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, and Pb. The blank for the cation exchange analysis was the NaCl exchange solution, which was used to correct the exchange cation values. The LB composition was compared with and without AMZ.

Minimum Inhibitory and Minimum Bactericidal Concentrations (MIC and MBC). The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) are defined as the concentrations of antimicrobial agents that reduce bacterial growth by 50% or 99.9% respectively, when compared to a control. To determine the MIC and MBC of the AMZ clay, a viability test was performed, varying both the clay concentration and the growth media (e.g., isotonic solution, NaCl, 0.85 %, and LB at 5 g/L and 25 g/L concentrations).

MIC/MBC of Al and Cu. To measure the MIC and MBC of metals of interest (e.g., Al, Cu) at a pH close to that buffered by the AMZ clay (i.e., 4.5–4.7), the viability

test was modified. Because certain metals can precipitate in neutral growth media pH (7.0–8.0), the LB was acidified by adding 0.1M HCl.

A concentrated stock of metal solution of 50 mM $AlCl_3$ and 10 mM $CuCl_2$, was sterilized by filtration (0.2 μm; Genemate™ vacuum filtration system). *E. coli* was grown to exponential phase in 5 g/L LB. Aliquots of 1 mL fresh culture (10^8 CFU/mL) were transferred into micro centrifuge tubes and cells were pelleted (4000 rpm, 3 min). After harvesting the cells, the neutral LB was discarded and cells were re-suspended in the acidified LB, followed by addition of dilutions of the metal stock to a final concentration of 0.5, 1.0, and 2.0 mM Al, and 0.05, 0.5, and 1.0 mM Cu. A control population of *E. coli* incubated in the acidified LB was included. All experiments were performed in triplicate. The pH and Eh was measured in the *E. coli* cultures after 24 h, using a Thermo Scientific ORION Dual Star meter.

Metal cocktail. To test for the effect of a consortia of metals derived from the AMZ, a metal cocktail was tested with the chloride salts of metals at the concentrations measured in the LB leachate (LB with AMZ; see Table 4.3). Because the individual concentrations of metals are low, the metal cocktail stock was prepared 100 times more concentrated and then diluted to the desired concentration representing that measured in the cation exchange solution. The metal cocktail stock contained 133 mM $AlCl_3$, 10 mM $MnCl_2$, 2 mM $FeCl_3$, 5 mM $CuCl_2$ and 4 mM $ZnCl_2$ mixed in Milli-Q water (18.2 MΩ-cm at 25 °C). The stock was filter-sterilized (<0.2 μm filter; Genemate™ vacuum filtration system) and stored at 4°C.

To test the antibacterial effect of the metal cocktail, a fresh sub-culture of *E. coli* (10^8 CFU/mL) was resuspended in acidified LB (pH 4.8). Then, 990 µL of the fresh culture was mixed with 10 µL of the metal cocktail. The culture was incubated with the metal cocktail overnight (37 °C) and bacterial viability was determined by standard plate counts. The pH and Eh of the culture exposed to metals were measured after incubation. To determine MIC and MBC, the metal cocktail was diluted 1:100, 1:500, and 1:1000 times, into the liquid culture (previously in acidified LB pH 4.8). To account for the effect of the acidified LB, a control of *E. coli* was incubated in acidified LB. The concentrations of individual metals in the mixture, at the MIC and MBC concentrations found, were calculated according to the dilution of the stock (e.g., $C_1V_1 = C_2V_2$). The metal speciation of the cocktail, at different concentrations was modeled using EQ3 software (Wolery, 1992) using the pH and Eh measured after 24 h of incubation with *E. coli.*

Nano Secondary Ion Mass Spectrometry (NanoSIMS). The ion distribution was investigated on cells using NanoSIMS (Li et al., 2008; Morrison et al., 2016; Orphan & House, 2009) Ion images of $^{12}C^{14}N$, $^{27}Al_2^-$, $^{56}Fe^{16}O^-$, and $^{63}Cu^-$ were collected on *E. coli*, incubated with AMZ clay via NanoSIMS and compared to untreated control *E. coli.* First, 10^8 CFU/mL cells in log phase growth were incubated in 5 g/L LB with a bactericidal dose of AMZ (100 mg clay/mL), followed by separation of the bacteria from clay using Nycodenz (Neveu et al., 2014; Londoño & Williams, 2016; Poté et al., 2010). The recovered *E. coli* was then fixed in 2 % glutaraldehyde. The samples were dehydrated by subsequent 5 min washes in 25, 50, 75, and 100 % ethanol. An aliquot of

one microliter was spotted onto a silicon wafer and allowed to dry for ion imaging via NanoSIMS (Morrison et al., 2016; Orphan & House, 2009).

Samples were imaged on the Cameca NanoSIMS 50L at the Arizona State University National SIMS Facility. A 15 μm^2 area was first pre-sputtered using a 30.5 pA current (dwell time = 1 μsec) to remove surface contamination (Hoppe et al., 2013). A primary Cs^+ ion beam was used, which can give a spatial resolution < 50 nm with a primary current of ~2 pA. The Cs^+ primary ion beam generates higher counts than the alternative O^- or O^{2+} primary beams. Therefore, negative secondary ions were detected. Some elements produce higher secondary signals for molecular ions. For example, Al_2^- at mass/charge (m/z) = 54 ionizes better than $^{27}Al^-$. The molecular ion $^{56}Fe^{16}O^-$ also gives higher counts than $^{56}Fe^-$, and $^{12}C^{14}N^-$ ionizes better than $^{12}C^-$ (Wilson et al., 1989). Only $^{63}Cu^-$ was measured as the major atomic ion. The data was processed using the CAMECA WinImage software (Cameca 2008, version 2.0.7). The images obtained consisted of a series of 6-10 planes representing different depths that were corrected for drift. Regions of interest (ROI) were drawn around *E. coli* cells to obtain total counts over the ROI. In addition, the planes of ion images were accumulated to compare the interior vs. membrane ion ratios (Hoppe et al., 2013). Ion ratios were calculated from the ROIs, with $^{27}Al_2$, $^{56}Fe\ ^{16}O$, and ^{63}Cu expressed relative to the matrix, i.e., the $^{12}C^{14}N$ signals from the cells.

Independent-sample t-tests were conducted in Microsoft Excel to compare the ion ratios in:1) the membrane of the *E. coli* treated with AMZ and the control *E. coli* membrane; 2) the interior of the *E. coli* treated with AMZ and the control *E. coli* interior*;*

3) the membrane and the interior of AMZ treated *E. coli*; and 4) the membrane and interior of control *E. coli.* The significance level was $p < 0.005$ for all.

Hydrogen peroxide production. Mineral surfaces and dissolved metals can produce H_2O_2 through Fenton reactions (Imlay et al., 1988; Nel et al., 2006, Schoonen et al., 2006). Hydrogen peroxide (H_2O_2) can react with transition metal ions, such as Fe^{2+}, to generate deleterious reactive hydroxyl radicals (•OH; Cohn et al., 2005). To quantify the production of H_2O_2 by clay, a method modified from Cohn et al., (2005) was used. Leuco-crystal violet (LCV) oxidizes in the presence of horseradish peroxidase (HRP) and H_2O_2 to form a crystal violet compound. The color change intensity is measured with a UV/VIS spectrophotometer at $\lambda = 590$ nm and converted to μM of H_2O_2 using a calibration curve, made by measuring the absorbance of known concentrations of H_2O_2. Briefly, AMZ clay was mixed with 0.85 % NaCl (isotonic solution) at a concentration of 80 mg clay/mL and incubated at 37 °C (in triplicate). After 2 h, an aliquot of the suspension was centrifuged at 16,000 rpm for 5 min to sediment the clay. 700 μL of supernatant was transferred into a 1.5 mL centrifuge tube and the following reagents were added: 200 μL 0.5M KH_2PO_4 buffer (pH 4.1), 48 μL LCV, 2 μL 0.5M EDTA, and 50 μL HRP. The samples were incubated at room temperature, in the dark, for 20 minutes before determining absorbance at 590 nm. A blank with isotonic solution and the reagents was included. The procedure was repeated after 6 h and 20 h of incubation. The H_2O_2 production from a non-antibacterial kaolinite KGa-1 was measured for comparison.

Testing membrane permeabilization. To test whether the AMZ clay increased the permeability of the outer membrane of *E. coli*, a test was performed using

vancomycin (Gordon et al., 2010). Vancomycin is a large antibiotic molecule that cannot pass through the porin channels of *E. coli* (Chen et al., 2009). However, if the outer membrane is breached, vancomycin passes through the outer membrane and inhibits the synthesis of the peptydoglycan membrane, therefore destroying the cell. Vancomycin hydrochloride from *Streptomycis orientalis* (Sigma-Aldrich) was diluted in sterile water to a concentration of 40 µg/mL. The viability test was conducted by mixing vancomycin with a sub-lethal dose of AMZ clay (50 mg/ml). The *E. coli* viability with AMZ plus vancomycin was compared with the individual effects of AMZ and 40 µg/mL vancomycin (See Appendix E for more details).

A one-way ANOVA (Bliss, 1967) was conducted in Microsoft Excel to compare the effect of incubating *E. coli* with vancomycin, *E. coli* with AMZ clay, and *E. coli* with both AMZ clay and vancomycin. Significance was established at $p < 0.05\%$.

Results

Clay Characterization

The clay fraction of AMZ contained over 82 % clay minerals and 15 % quartz identified by random powder X-ray diffraction and quantified by Rock Jock (Fig. 4.1; Table 4.1). The clay minerals were primarily 1:1 kaolins (29 % kaolinite and 15 % halloysite), and 2:1 clay minerals (30 % smectite), as shown in Table 4-1 and in Table 3.1 (see also Londoño & Williams, 2016).

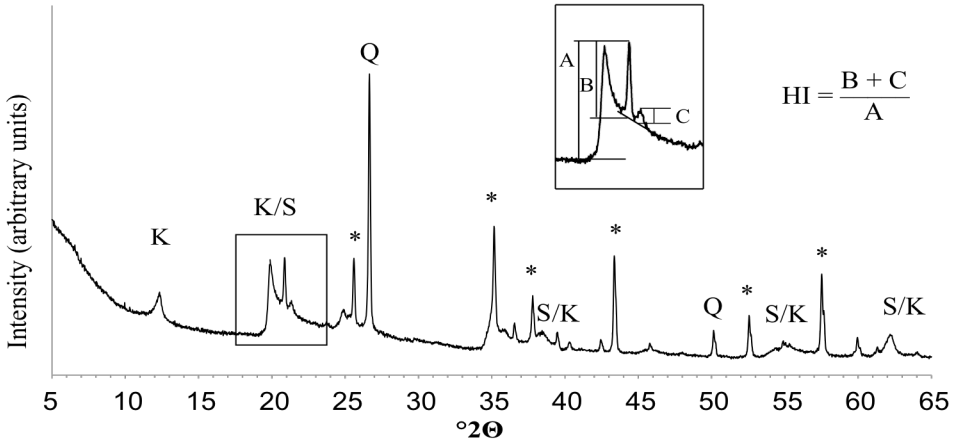

Figure 4.1 Interpreted XRD with the most common minerals of the AMZ and calculation of the Hinckley Index (HI). K, kaolin group minerals; Q, quartz; S, smectite; I/S, superposition of illite and smectite reflections; S/ K, superposition of smectite and kaolinite reflections * α-alumina standard. Full pattern degree of fit= 0.0342. Figure modified from Londoño & Williams, 2016.

Table 4.1

Quantitative Mineralogy of the AMZ

Mineral	Wt %
Quartz	15
Anatase	0.4
Clay minerals	
Halloysite	15.4
Kaolinite	29
Smectite	30
Illite	0.6
Muscovite	6.9
Total clays	81.8
Total Mineral	97.9

Note. Determined with Rock Jock software (Eberl, 2003)

The structural defects of the AMZ clay were assessed using the Hinckley index, as shown in Figure 4.1. The HI of AMZ is 0.8, which indicates a moderately disordered structure; the HI can range from ~0.2 to 1.5, with the larger values indicating greater "crystallinity" (Hinckley, 1962; Table 4.2).

Due to the presence of both smectite and kaolinite in the AMZ, the total clay surface area (TSSA) and the cation exchange capacity (CEC) fell between the range of values for the reference smectite (SWy-1) and of the reference kaolinite (KGa-1; Table 4.2; Londoño & Williams, 2016 Table 3.3). However, the AMZ clay had a higher specific surface area (51.2 m^2/g) than either of the reference clays.

The minerals in the AMZ had a variety of morphologies including vermicular or stacked, flaky, and curled (Figure 4.2). The surface of the AMZ clay was generally rougher than that of the reference smectite (SWy-1) and the reference kaolinite KGa-1, which is a well-ordered kaolinite (HI = 1.0). The SEM images of AMZ clay in Figure 4.2, show the different surface and clay morphologies present.

Table 4.2

Characteristics of the AMZ and Two Reference Clays

	Total Surface Area[a] (m^2/g)	Specific Surface Area[b] (m^2/g)	Cation Exchange Capacity (meq/100g)	Hinckley Index (HI)
SWy-1	698.8	29.8	76.7	N/A
KGa-1	45.2[c]	10.0[d]	2.0[d]	1.0
AMZ	278.8	51.2	29.3	0.8

Note. a) TSSA Determined by ethylene glycol monetlyl ether (EGME). b) Determined by the Brunauer, Emmett & Teller method (BET). Data from c) Kennedy & Wagner (2011) d) Olphen & van Fripiat (1979). N/A, the HI does not apply to smectites.

The vermicular kaolinite (Figure 4.2a), shows a stack of pseudo-hexagonal platelets with a smooth surface, in contrast with the kaolinite in Figure 4.2b, which displays a rough surface. Flaky smectite shows a curly cornflake texture (4.2c), and possible nanotubes of halloysite (H) appear on top of the flakes. Figure 4.2d shows platy kaolinite (PK) and other crystals with irregular shapes possibly indicating dissolution textures (DT).

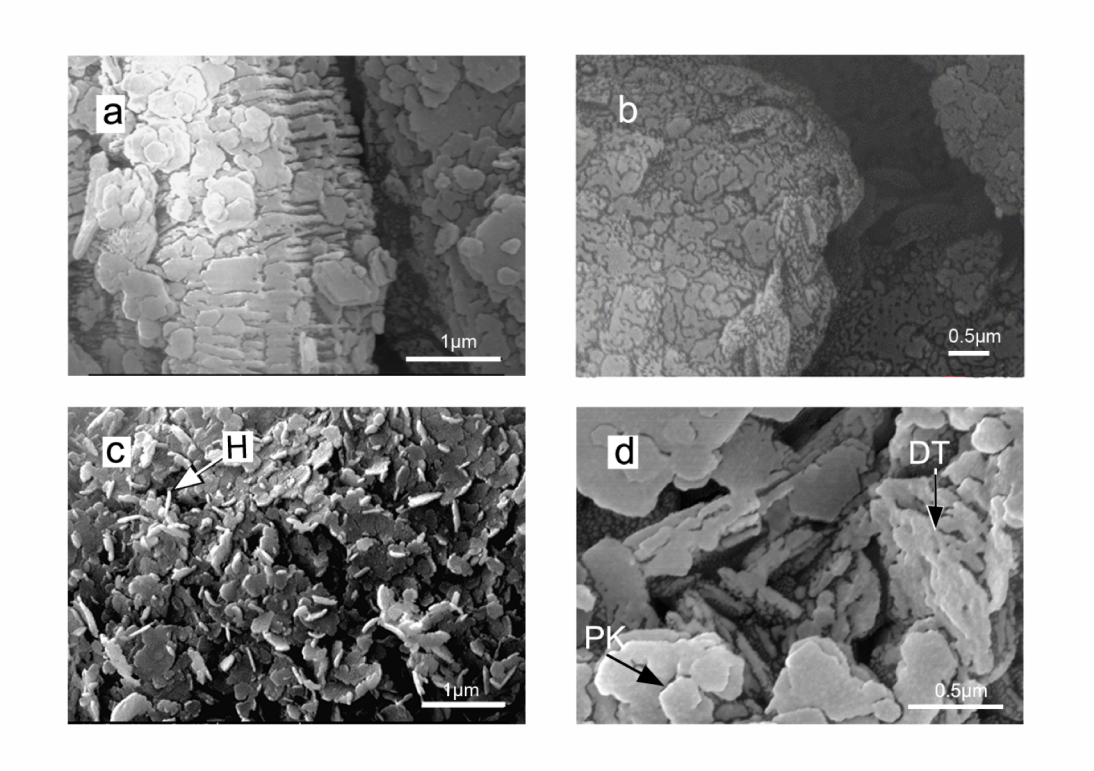

Figure 4.2 Different morphologies of the AMZ kaolins observed with Scanning Electron Microscopy. A) vermicular kaolinite, b) dissolution texture on kaolin surface c) flaky smectite (H. halloysite?), d) dissolution textures (DT) contrast with well formed pseudo-hexagonal kaolinite (PK) with smooth surface.

Antibacterial Activity of the AMZ Clay

The rate of *E. coli* death when incubated with 80 mg/mL AMZ (killing curve) was determined in isotonic solution (0.85 % NaCl). DI water causes osmotic shock so isotonic solution was used as a proxy for a 'no nutrient' solution. Figure 4.3 shows that *E. coli* cells treated with AMZ were no longer viable after 8 h of incubation, compared to the control without clay in isotonic solution.

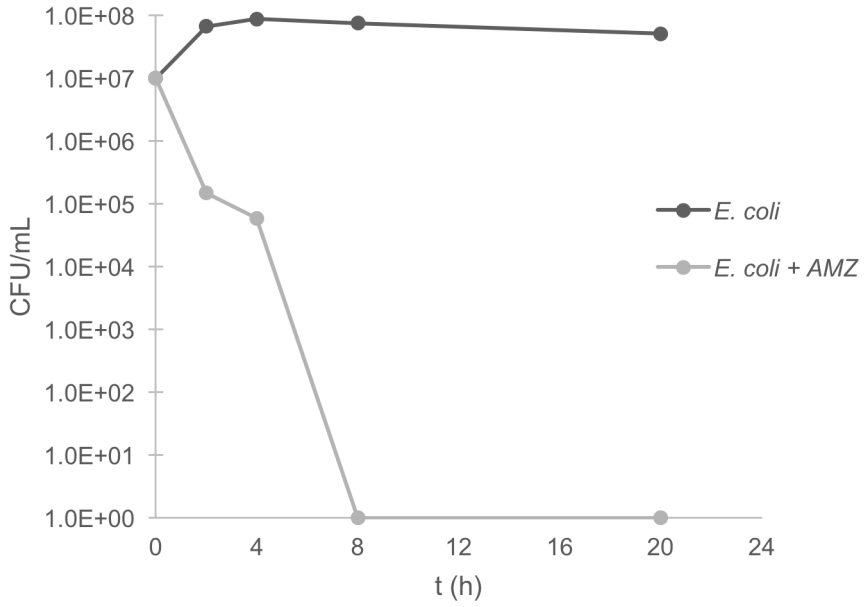

Figure 4.3 Kill curve in isotonic solution (80 mg/mL) compared to that for *E. coli* incubated in isotonic solution alone. Note that the rate of cell death is faster for the *E*. coli + AMZ treatment.

The minimum inhibitory and minimum bactericidal concentration (MIC & MBC) for the AMZ clay was determined in different growth media, which affects the nutrient availability and ion speciation. For example, 50 mg AMZ/ml isotonic solution reduced 50 % of *E. coli* population (MIC), while 75 mg/mL was required in 5 g/L LB and 250 mg/mL of AMZ clay was the MIC in 25 g/L LB. These results show that more clay is needed to inhibit *E. coli* when bacteria are cultured in complex media containing a mixture of salts and organics (i.e. tryptone and yeast extract), such as LB, rather than in isotonic NaCl solution (Table 4.3).

Table 4.3

MIC and MBC Range for AMZ Clay in Different Media

Media	MIC (mg/mL)	MBC (mg/mL)
0.85 % NaCl	50	90
5 g/L LB	75	100
25 g/L LB	250	300

Note. Initial culture concentration 10^8 CFU/mL

Effect of Low pH on *E. coli* Viability

The pH of the media incubated with AMZ changed over 12 h. The pH of 5 g/L LB incubated with the AMZ increased from 4.5 to 4.7. The overall acidic conditions can compromise the stability of the *E. coli* cell membrane (Beveridge & Koval, 1981; Borrok, 1999), therefore viability loss caused by a pH 4.5 buffer was compared to the viability loss caused by the MBC of AMZ clay. A 0.1 M citric acid-citrate buffer was mixed with 5 g/L LB. The buffered LB had a pH of 4.5 and reduced the viability of the *E. coli* by three orders of magnitude compared to controls grown without the buffer. Yet, 100% of

the *E. coli* population was killed after incubation with the100 mg/ml AMZ (Figure 4.4), a reduction in viability, which surpasses that caused by pH 4.5 alone.

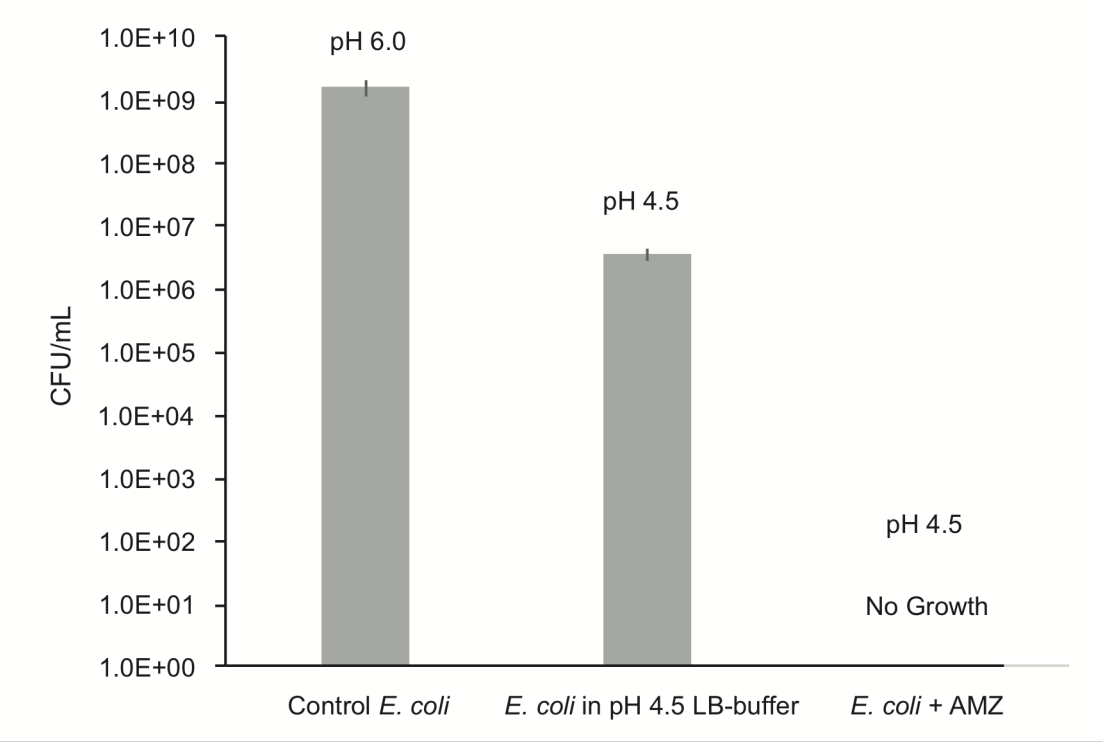

Figure 4.4 Viable colonies as a function of pH and experimental treatments. *E. coli* incubated in pH = 4.5 buffered LB media lost its viability by three orders of magnitude, while *E. coli* incubated with the AMZ clay, at the same pH lost 100 % of its viability. Error bars correspond to one standard deviation of the mean of three

Chemical Changes in AMZ Clay after Incubation with *E. coli*

The incubation of bacteria with the AMZ clay changes the concentration of the elements in the minerals, the *E. coli,* and the media. The chemical changes in *E. coli* were studied in Chapter 3 (Londoño & Williams, 2016, see Table 3.7), but here the changes in the AMZ chemical composition after incubation with *E. coli*, are examined. In addition, a chemical analysis of a cation exchange solution of AMZ provides new data about the

potential chemical transfer of exchangeable ions from the clay to bacteria. For this discussion, the clay that was incubated with *E. coli* will be referred to as 'reacted-AMZ', and the AMZ without incubation as the 'control AMZ'. Table 4.4 shows the chemical changes in the reacted-AMZ, compared to control-AMZ.

Table 4.4

Chemical Composition (ppm) of AMZ Clay without and After Incubation with E. coli

	Control AMZ	SD	Reacted AMZ	SD	Exchange solution AMZ	SD
Mg	3595	134	2259	126	45.0	0.5
Al	141,138	1,271	103,625	3,469	108	1
P	150	3	1117	78	BDL	N/A
K	9167	388	7171	385	19.9	0.4
Ca	1348	28	454	41	98	1
Ti	4061	141	3218	291	0.16	0.02
V	152	6	116	10	0.0021	0.0001
Cr	89	3	66	5	0.01	0.0002
Mn	130	5	41	3	9.44	0.0740
Fe	22084	917	16486	1196	0.31	0.02
Co	5.6	0.2	2.6	0.2	0.329	0.002
Ni	14.2	0.5	8.9	0.6	0.27	0.003
Cu	82.5	2.8	52.1	4.1	4.87	0.03
Zn	46.3	1.6	31.5	2.5	1.03	0.01
As	3.2	0.1	2.3	0.1	0.04	0.001
Se	12	1	7.7	0.6	–	–
Rb	80	4	49.6	17.5	–	–
Sr	85	3	53.6	2.1	–	–
Zr	101	4	72.9	3.0	–	–
Mo	4.2	0.2	3.5	0.2	–	–
Ag	0.115	0.005	0.08	0.01	–	–
Cd	0.1	0.00	0.03	0.00	–	–
Ba	225	7	157	8	–	–
Pb	25.6	0.7	20.4	1.4	0.29	0.003

Note. Exchange solution was 1N NaCl equilibrated with 100 mg AMZ/mL for 24 h.
BDL, below detection limits, (–) not measured, N/A, Not Available.

In the reacted–AMZ, the concentration of P increased to 1117 ppm compared to the control AMZ (150 ppm P); but phosphorus is not detected in the exchange solution, indicating that AMZ is adsorbing P. The dominant exchangeable cations in the AMZ are: Al (108 ppm), Ca (98 ppm), Mg (45 ppm) and K (20 ppm), followed by transition metals,

such as Mn, Cu and Zn (< 10 ppm; Table 4.4). This indicates that these elements were held in exchangeable sites of the clay minerals (kaolinite, halloysite, and/or smectite) assuming no dissolution. Notably, the concentrations of all these elements decreased in the reacted–AMZ compared to the control–AMZ, but the change in the amount of Al and Fe far exceeds the amount in the exchange sites, with a loss of 34000 ppm Al and 6000 ppm Fe in the reacted-AMZ respectively. The increase in P in the reacted-AMZ could be either from P in the media or from P from in the cell phospholipid membranes. To test the hypothesis that the clay was absorbing P from the LB, AMZ was incubated with 5 g/L LB overnight at 37 °C (80 mg clay/mL representing the MIC). The LB incubated with AMZ lost 34 ppm of phosphate compared to the control, leaving only 6 ppm P in the media. Notably also the LB incubated with AMZ gained 36 ppm Al (Table 4.5).

Table 4.5

Chemical Concentrations (ppm) of Growth Media (LB) and LB Incubated with AMZ

	LB[a]–No Clay	SD	LB with AMZ[b]	SD
Na	578	17	545	4
Mg	0.82	0.02	30	2
Al	0.08	0.001	36	11
P	40	3	6	1.0
K	120	2	101	2.9
Ca	1.84	0.1	63	2.4
V	0.01	0.0004	0.12	0.04
Mn	0.001	0.00005	6.1	0.3
Fe	0.12	0.002	0.97	0.2
Co	0.009	0.0007	0.23	0.01
Ni	0.002	0.0001	0.23	0.01
Cu	0.06	0.002	3.4	0.5
Zn	0.30	0.02	3.0	0.09
As	0.0005	0.00001	0.01	0.002
Se	BDL	–	0.1	0.02
Rb	0.054	0.004	0.1	0.01
Sr	0.012	0.0009	1.23	0.03
Ba	0.060	0.004	1.60	0.12
Pb	0.003	0.0002	0.03	0.03

Note. a) 5 g/L LB. b) Clay concentration was 80 mg/mL. BDL is below detection limit

To test whether the decreased P in the LB media, in the presence of AMZ, limited bacterial growth, *E. coli* was incubated with a sub-lethal concentration of AMZ (70 mg/mL in 5 g/L LB) for 2 h, and then the growth media was supplemented with 2 mM sodium phosphate monobasic (NaH_2PO_4), this concentration was chosen to double the amount of P removed by clay adsorption. Controls consisted of *E. coli* incubated in 5 g/L LB without clay, and *E. coli* incubated in 5 g/L LB with AMZ, but without adding P. Results indicate that the *E. coli* population declined by three orders of magnitude regardless of the addition of PO_4^{3-} (Figure 4.5).

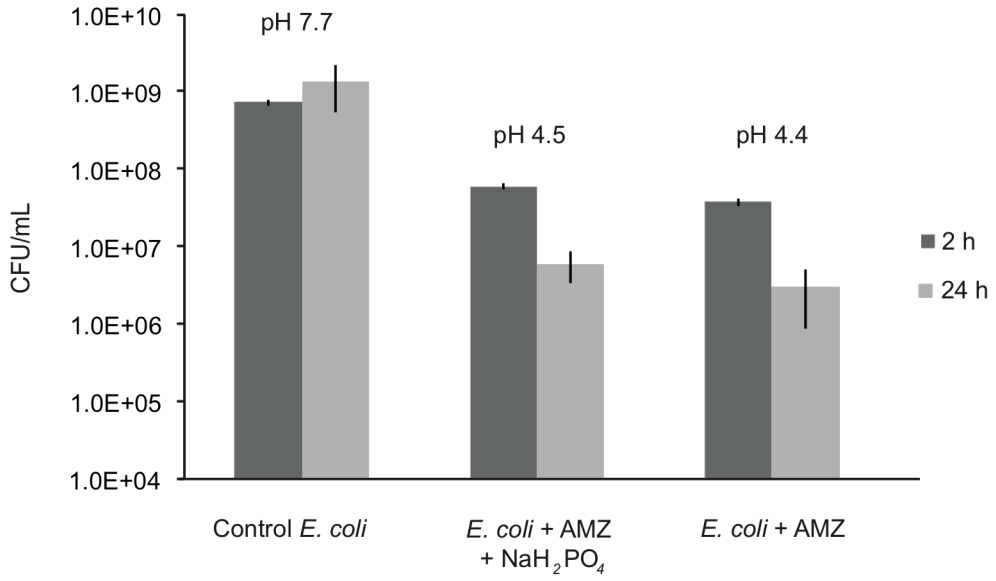

Figure 4.5 Viable colonies of *E. coli* incubated for 2 h with AMZ and supplemented with 2 mM phosphate. The addition of NaH_2PO_4 did not change the viability loss produced by the AMZ alone. Error bars correspond to one standard deviation of the mean of three measurements.

Effect of metals.

Of all the elements, Al consistently increased in the LB incubated with AMZ (36 ppm), in the exchange solution (108 ppm), and in *E. coli* reacted with AMZ (Table 3.7; Londoño & Williams, 2016). Cu, a transition metal known to have antibacterial properties (Kimura & Nishioka, 1997; Macomber & Imlay, 2009; Rodriguez–Montelogo et al., 1993), also increased slightly in the LB when incubated with AMZ (3.4 ppm; Table 4.5) and in the exchange solution (~5 ppm; Table 4.4). The MIC and MBC of Al and Cu were determined at pH 4.5, the pH buffered by the AMZ clay (Table 4.6).

Table 4.6

Minimum Inhibitory and Minimum Bactericidal Concentrations (ppm) of Al and Cu at pH 4.5

Element	MIC	MBC
Al	13.5	54
Cu	6.4	63.5

Results showed that the concentration of Al in the LB incubated with AMZ is ~2.5 times above the MIC. The Al concentration in the exchange solution (108 ppm) is double that of the MBC, however the exchange solution does not represent the levels to which bacteria is exposed, rather it represents the total concentration of elements available for exchange. Because the aqueous leachate of AMZ contained less Al than required for bacterial growth inhibition, the hypothesis is that exchangeable cations such as Al may be transferred during bacterial contact with the hydrated clay.

The Cu concentration in the LB incubated with AMZ is below MIC concentration by ~half. It was previously determined that the MIC of Fe at pH 4.5 in LB, is between 167–275 ppm (Morrison, 2016) depending on LB concentration, however for AMZ the concentration of Fe did not exceed 1 ppm in the LB incubated with AMZ, nor in the exchange solution. Therefore, the Cu and Fe *alone* are not considered antibacterial.

However, given the abundance of exchangeable transition metals present in the AMZ, it is possible that combined they may play a role in the toxicity to *E. coli*. Therefore, to test the collective effect of transition metals at the pH of the experiment (~4.6) on *E. coli,* a solution of dominant metals found in the LB incubated with AMZ (Mn, Fe, Cu, Zn) was prepared with their chloride salts at the same concentrations measured. Table 4.6 gives the concentration of the transition metal cocktail, the Al concentration and the MIC and MBC that were calculated based on the dilution factor. Note that the first column matches the concentrations found in the 5 g/L LB incubated with AMZ (Table 4.5).

Table 4.7

Concentration, MIC50, and MBC in (ppm) for the Metal Cocktail

Element	Metal cocktail[a]	MIC in mixture	MBC in mixture
Al	35.8	3.6	7.2
Mn	5.5	0.5	1.1
Fe	1.1	0.1	0.2
Cu	3.2	0.3	0.6
Zn	2.6	0.2	0.5
pH	4.0	4.5	4.7
Eh (mV)	252	243	223

Note. a. The concentrations correspond to those measured in LB reacted with AMZ. The MIC and MBC values were calculated from the dilution. pH and Eh measured after 24 h of incubation

The *E. coli* did not survive incubation with the metal cocktail at the concentrations found in the LB with AMZ (Figure 4.6). The MBC of the metal cocktail was achieved by the diluting the mixture 5 times, and the MIC by diluting it 10 times (Figure 4.6). The Al concentration in the metal cocktail, in all the dilutions, was inhibitory, not bactericidal suggesting that the antibacterial effect of the metal mixture is greater than the effect of Al alone.

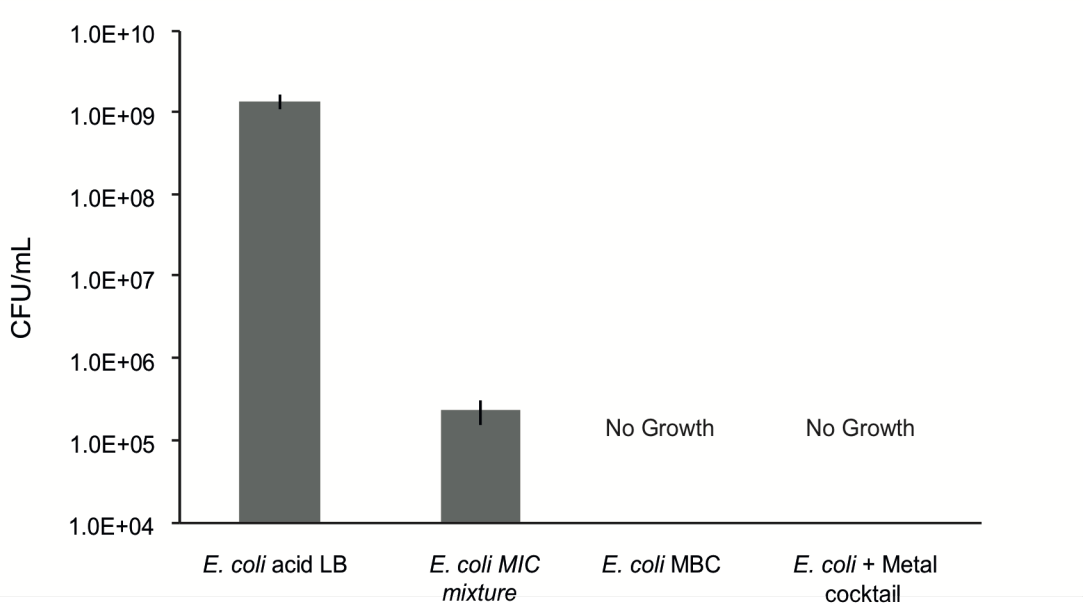

Figure 4.6. Viable colonies for *E. coli* exposed to MIC and MBC metal cocktail concentrations and to the concentrations in the LB incubated with AMZ. The control *E. coli* cultivated in acid LB grew to 10^9 CFU/mL after 24 h. The pH values reported were measured after 24 h incubation period. All the experiments had a pH of 4.6 Error bars represent the standard deviation of three independent experiments. Error bars correspond to one standard deviation of the mean of three measurements.

All the experiments had an initial pH of 4.6, but after 24 h of incubation, the pH had changed (Table 4.7). The pH of LB in the control *E. coli,* increased to 6.5 with an Eh of 156 mV, in the absence of metals derived from AMZ clay.

Metal Speciation

Metal toxicity is heavily influenced by pH, which controls metal speciation. Table 4.8 shows the aqueous speciation of the metal cocktail and its different dilutions was modeled using EQ3 (Wolery, 1992) at the concentrations and aqueous conditions (pH, Eh) shown in Table 4.7.

Table 4.8

Aqueous Metal Speciation and Concentrations (ppm) Calculated Using EQ3 at the pH and Eh Measured.

Element	Species	Metal cocktail[a]	MIC	MBC
Al	Al^{+3}	30.9	1.9	2.7
	$AlOH^{+2}$	4.6	1.2	2.5
	AlO^{+}	0.3	0.3	1.1
	$HAlO_{2,AQ}$	–	0.1	0.6
	Total Al	35.8	3.5	7.0
Mn	Mn^{+2}	6.03	0.56	1.12
	Total Mn	6.03	0.56	1.12
Fe	Fe^{+2}	0.77	0.11	0.22
	$FeOH^{+2}$	0.17	–	–
	Total Fe	0.94	0.11	0.22
Cu	Cu^{+2}	3.10	0.29	0.53
	Cu^{+}	–	0.03	0.10
	Total Cu	3.10	0.32	0.63
Zn	Zn^{+2}	2.97	0.26	0.05
	Total Zn	2.97	0.26	0.05
Cl	Cl^{-}	153.4	3.5	22.0
	Total Cl^{-}	153.4	3.5	22.0

Note. a) Metal cocktail at the concentration found in LB incubated with AMZ (Table 4.7).

The dominant Al species present was Al^{+3}, but as the pH increases towards pH 5.0, $AlOH^{+2}$ is the dominant stable specie. The transition metals such as Mn and Zn were

present in their +2 state in all the solutions, and also the majority of the Cu and Fe. The combination of these species appears to be toxic to bacteria.

NanoSIMS results

Isotopic distribution maps were generated by NanoSIMS which indicate the location of various elements concentrated in the *E. coli* treated with AMZ. Of interest is whether the predominant Al species in solution are affecting the cell membrane or intracellular components. Monitoring the distribution of $^{12}C^{14}N$ outlines the dominant matrix of the *E. coli* (Figure 4.8). Calculating the ratio of the metal to the matrix is a standard way to compare the relative abundance of elements in cells (Hoppe et al., 2013; Orphan & House, 2009).

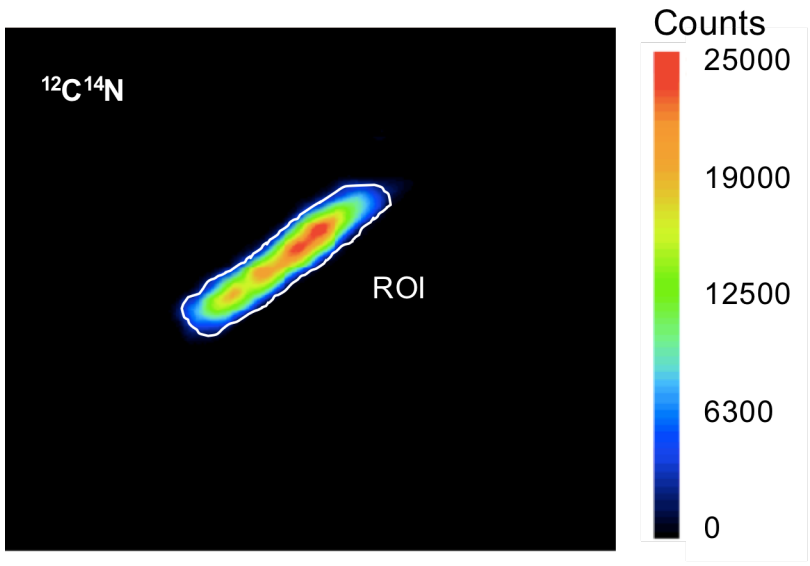

Figure 4.7 Example of an isotope map of $^{12}C^{14}N$ in *E. coli.* A total of 6 images were produced, corresponding to different depths. The planes were accumulated (added) and combined in sets representing regions of interest (ROI) in *E. coli.*

The data obtained is not quantitative, because it requires a standard of each element in a similar matrix. However, the Al/CN and Fe/CN ratios show a qualitative increase in *E. coli* treated with AMZ relative to the control–*E. coli*. The counts of Cu/CN were too low to achieve statistical significance (Figure 4.8).

To further investigate the distribution of ions in three dimensions through the bacteria, planes of data collected at different depths through the cell were accumulated to compare the ion counts in the cell interior to those in the lower cell membrane (data collected just before the cells were sputtered away). This approach eliminates the topographic effects on ion sputtering, an artifact that occurs due to the short-working distances between the primary ion beam, the sample, and the instrument optics (Orphan & House, 2009). Data were accumulated from these regions of interest in 3 AMZ treated vs. 3 control cells.

Results showed that Al/CN is higher in both the interior and the membrane of the AMZ treated *E. coli*, compared to the control, and that the cell membrane has higher Al than the interior (Figure 4.8). Within the AMZ treated cells, the difference between Al in the membrane and the interior was significant ($p = 0.006$), and Al was greater in the membrane. Conversely, the Fe/CN is greater in the interior of *E. coli* reacted with AMZ compared to the control ($p = 0.001$), and it is significantly higher than in the control *E. coli* (Figure 4.8, Table 4.8). No significant difference was detected for Cu/CN between the control and the AMZ treated *E. coli* (Figure 4.8) due to the low signal.

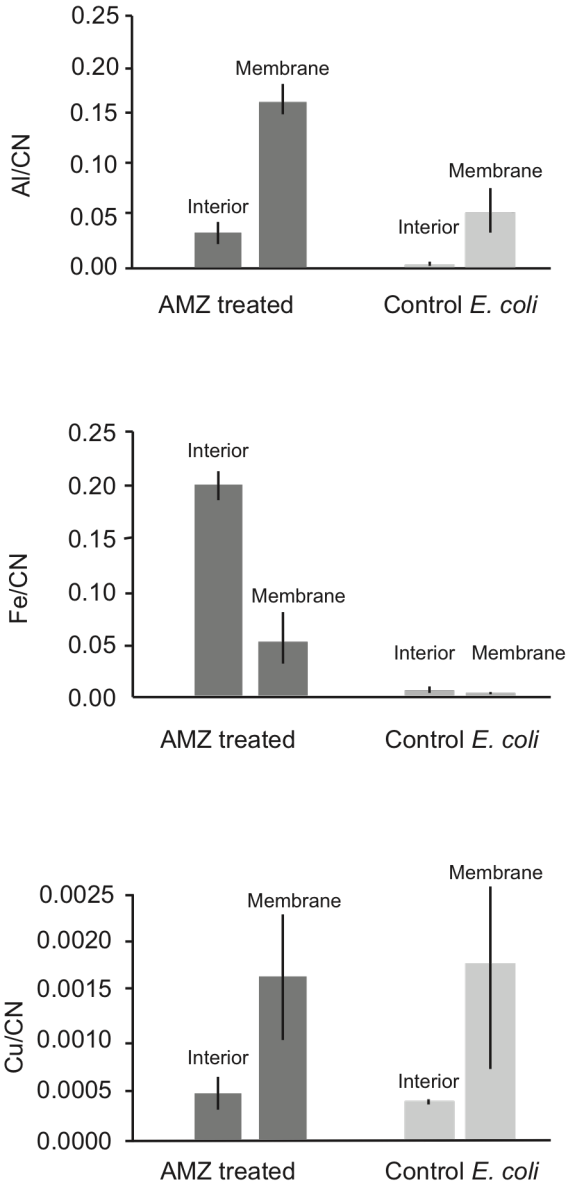

Figure 4.8 Isotope ratios of accumulated signals from rastered planes in E. *coli*. Data collected from the middle of the cell (interior) is compared to data collected from the bottom membrane of the cell in AMZ treated *E. coli* and control *E. coli.* Error bars correspond to one standard deviation of the mean of three measurements

Table 4.9

p value from t-test

	Al/CN	Fe/CN	Cu/CN
Membrane (AMZ vs. Control)	*0.004*	*0.2*	*0.9*
Interior (AMZ vs. Control)	*0.01*	*0.001*	*0.7*
AMZ (Interior vs. Membrane)	*0.007*	*0.03*	*0.1*
Control (Interior vs. Membrane)	*0.07*	*0.2*	*0.2*

Note. p value from t-test, 2 tailed test, equal variance. Significance criteria $p < 0.05$

Hydrogen Peroxide Production

Hydrogen peroxide, produced from reduction of O_2 during oxidation of reduced metals, is a precursor to formation of reactive oxygen species (ROS). H_2O_2 production can be used as a proxy to evaluate ROS production, as it is an intermediate species required for ROS production. Using the leuco-crystal violet method developed by Cohn et al. (2005), 80 mg/mL of AMZ clay produced sub-micromolar concentrations of H_2O_2

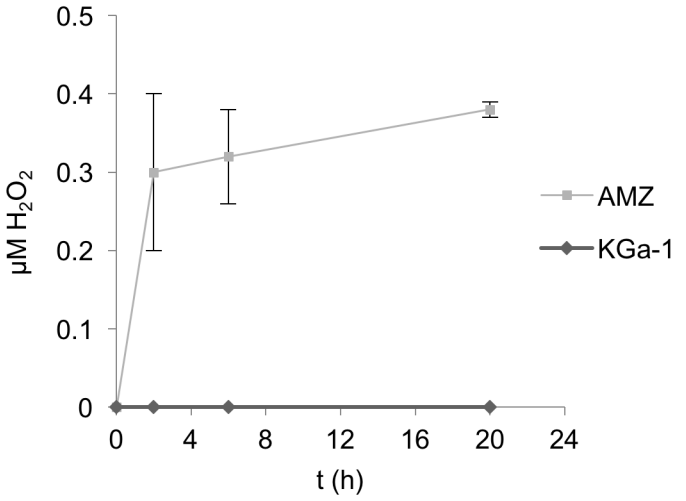

Figure 4.9 Production of H_2O_2 vs. time by AMZ (80 mg/ml) and by the reference kaolinite (KGa-1; 80mg/mL), both in isotonic solution. Error bars correspond to one standard deviation of the mean of three measurements.

(Figure 4.9). The H_2O_2 increased rapidly from 0 to 0.3 μM over 4 h, but then reached a metastable plateau at 0.4 μM after the 20-hour experiment. The control kaolinite did not produce H_2O_2 during the 20 h of the experiment.

Membrane Permeability

To test whether the AMZ clay treatment increases the permeability of the outer membrane of *E. coli* a test was designed using an antibiotic that would only attack *E. coli* if its outer membrane failed to protect it. Vancomycin, an antibiotic used against Gram-positive bacteria, was used because it inhibits the peptidoglycan (PG) layer synthesis, between the inner an outer membrane of *E. coli*. Contrary to Gram-positive microorganisms, the outer membrane in *E. coli* protects the PG layer, thus the action of vancomycin is very limited in *E. coli*. The premise of this experiment is that the Al might

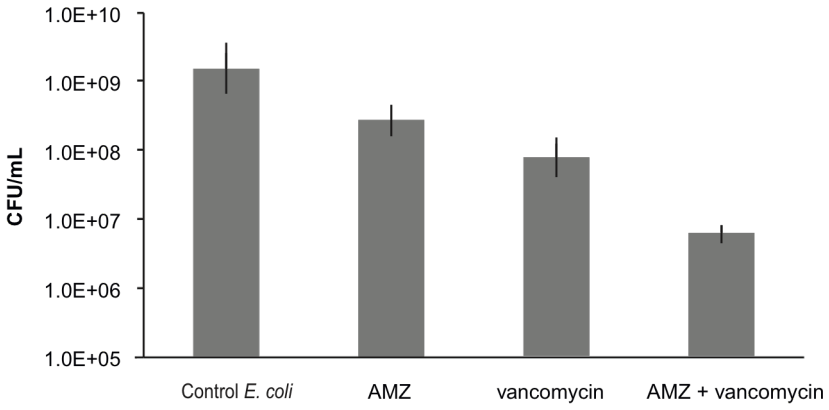

Figure 4.10 Viable colonies of *E. coli* after incubation with AMZ, vancomycin, and vancomycin plus AMZ. Error bars correspond to one standard deviation of the mean of three measurements. An analysis of variance showed significant differences among all the groups, $F(2,23) = 5.6$, $p < 0.05$.

compromise the outer membrane, allowing the vancomycin molecule to enter the cell and aid in further decreasing bacterial viability by damage to the PG layer.

Results showed that the viability of *E. coli* incubated with a sub-lethal dose of AMZ (50 mg/ml) plus vancomycin was two orders of magnitude less than the viability loss caused by 50 mg/ml of AMZ alone, and three orders of magnitude less than that shown by the control *E. coli* (Figure 4.10). The dose of vancomycin (40 µg/mL) plus AMZ had a significant effect on *E. coli* at the $p < 0.05$ level according to an ANOVA analysis of variance.

Discussion

Upon incubation with AMZ, *E. coli* faces a chemical environment that is mildly acidic (~pH 4.5), enriched with a combination of exchangeable ions and dissolved structural ions from the clay (e.g., Al, Fe, Cu), that can be bactericidal. Notably, the Al was above the inhibitory concentration in the LB media incubated with AMZ (Table 4.3 & 4.5), and it accumulated preferentially on the *E. coli* membrane (Figure 4.8 and Table 4.9). Excess Fe was found inside the AMZ–treated *E. coli* compared to that observed in the control *E. coli* (Table 4.9), and this was previously identified as an antibacterial mechanism for *E. coli* (Morrison et al., 2016), although requiring much higher concentrations than measured in this study. The experiment with a metal cocktail, that simulated the metal solution experienced by AMZ treated *E. coli,* indicated that the metal

mixture is more damaging to cells than individual metals and lower concentrations of metals can affect the cells viability when they are combined (Table 4.7).

Previously it was observed that P was depleted in AMZ-reacted clays (Table 4.4) and it was hypothesized that nutrient depletion contributed to bacterial death (Londoño & Williams 2016). The AMZ clay adsorbed 90% of the P from the LB (Table 4.4), but restoring the original concentrations of P in a culture incubated with AMZ, did not revive bacterial growth (Figure 4.5). This could indicate that the AMZ destroyed the cells rather than rendering them dormant. Peterson et al. (2005) showed that bacteria commonly enter stationary phase when threatened by P starvation, however if the *E. coli* were stationary they should have revived when P was supplied. *E. coli* can survive under very limited P conditions by both genetic and metabolic strategies and the 6 ppm P measured in the LB reacted with AMZ is enough to sustain *E. coli* metabolism (Lethola et al., 1999; Marzan & Shimizu, 2011; Peterson et al., 2005). Therefore, it is interpreted that the P loss from the bacteria does not reflect nutrient deprivation, but rather it corresponds with damage to the phospholipids in the cell membrane. These results do not support the hypothesis that phosphorous depletion causes viability loss in *E. coli*.

The AMZ clay is mostly composed of kaolins (45%; Londoño & Williams, 2016). The Hinckley Index (HI = 0.8) indicates that the kaolinite is moderately disordered. Alternatively, the HI indicates a mixture of kaolinites, each with a different amount of defects (Plançon et al., 1988). Mineralogical analyses (XRD) showed that clay minerals present in the AMZ are dominated by kaolinite, halloysite and smectite (Table 4.1; Table 3.1; Londoño &Williams, 2016). The external surfaces of kaolins and smectite have a

negative charge, but the edges of the clay minerals, where bonds are broken, have a pH-dependent charge (Moore & Reynolds, 1997). At low pH these charges are positive and can adsorb anions, such as PO_4^{3-} (Murphy, 1939) possibly explaining the gain in P in the AMZ incubated with *E. coli* compared to the control AMZ (Table 4.3) and the decrease in P in the LB incubated with AMZ (Table 4.4).

The presence of 30% smectite in the AMZ clay (Table 4.1, Table 3.1; Londoño & Williams, 2016), is likely responsible for the vast surface area of the clay assemblage (279 m^2/g TSSA and 51 m^2/g SSA, Table 4.2). The total surface area of smectites is generally greatest because of its interlayer, where the siloxane surface has a negative charge compensated by exchangeable cations. Halloysite, with a large lumen in its tubular structure, can hold a variety of exchangeable cations, anions, and neutral molecules. In the AMZ clay, a variety of transition metals, (e.g., Mn, Fe, Cu, Zn; Table 4.3 and 3.2; Londoño & Williams, 2016) may be present in exchange sites or may substitute as trace elements in the octahedral sites of smectites and kaolins. The concentration of these cations in the aqueous leachate is very low (Table 3.5, Londoño & Williams, 2016), which may explain why the leachate is not antibacterial. However, the cation concentration increased both in the Cl-rich exchange solution and in the LB incubated with AMZ (Table 4.3) implicating their involvement in the antibacterial process. The AMZ clay may release its metal cations via exchange or dissolution. Low pH (4.0) and the disorder in the mineral structure are factors that aid the clay dissolution process (Brantley et al., 1986; Lasaga & Blum, 1986; May et al., 1986; MacInnis & Brantley, 1992; Tsuzuki & Kawabe, 1983). The AMZ clay buffers the pH to mildly acidic pH (4.5) and has moderate defects. Furthermore, the texture of the AMZ surface

(Figure 4.2b, 4.2d, and 3.2) shows dissolution features therefore metals shown to affect the bacteria (e.g., Al, Fe, Cu) could be sourced from both exchangeable and structural (i.e., octahedral) sites of the AMZ.

Role of pH in the Antibacterial Process

Halloysite can concentrate H^+ due to the Al-OH^- groups in the lumen that attract cations (Theng et al., 1982). It is the balance of H^+ or OH^- to the clay surface that buffers the pH of the aqueous suspension of the AMZ to low pH and promotes adsorption of cations or anions in specific sites. The pH of the LB culture media of *E. coli* incubated with the AMZ clay varied between 4.5–4.6 after 24 h. The LB buffered to pH 4.5, acidified to assess the damage by protons, showed a decrease of *E. coli's* viability by 30 % (Figure 4.4), while the 70 % living cells indicate that the model strain of *E. coli* I used (ATCC 29925) can tolerate the mildly acidic environment. Furthermore, *E. coli* can grow in the pH range 4.4-9.2 (Stancik et al., 2002). Therefore, the proton concentration, or the damaged caused by protons, is not the sole cause of bactericide by the AMZ clay, as previously observed for antibacterial clays (Cunningham et al., 2010; Williams & Haydel, 2010). Yet, the antibacterial activity of the AMZ clay only proceeds at low pH. The pH of the environment, along with the oxidation state, ionic strength and temperature, determines the speciation of the metals. Thus, I surmise both metal speciation and concentration determine toxicity.

Metal Toxicity

Heavy metals are toxic to microorganisms at concentrations above their MIC or at lower concentrations, as this work has shown, in combination with other metals. Overall, the metal concentration increased in both the LB incubated with AMZ (Table 4.4), and in the *E. coli* incubated with AMZ (Table 3.7; Londoño & Williams, 2016). The metal concentration in isotonic solution was not measured, but the AMZ clay was antibacterial in water and isotonic solution as well (See Appendix D). Aluminum, with 36 ppm in the LB incubated with AMZ, is double the MIC (MIC = 13.5 ppm at pH 4.5, Table 4.5). Followed by Cu, which showed a concentration in the LB incubated with AMZ that is half of its MIC at pH 4.5 Other metals (e.g., Mn, Fe, As, Ni, Pb) are very dilute in the LB or the exchange solution compared to MICs reported in the literature, for example, the Fe in the LB incubated with AMZ is < 1ppm, but the MIC is between 167–275 ppm (for Fe^{3+} and Fe^{2+} respectively, in 10 g/L LB at pH 4.5; Morrison et al., 2016). Manganese is only toxic to *E. coli* at high concentrations (more than 1098 ppm, Nies, 1999); which were not exceeded in the LB incubated with AMZ (Table 4.4) nor in the exchange solution (6 and 10 ppm respectively, Table 4.3).

The metal cocktail, prepared with the five most abundant elements in the LB incubated with AMZ (Table 4.6) showed that the combination of Al, Mn, Fe, Cu, and Zn is bactericidal. If only the Al was antibacterial, the cockatil would have been inhibitory, rather than bactericidal, since the Al levels were below the MBC. This suggests that the combination of metals produces the bactericidal effect when combined. Metals can interact synergistically to produce a toxicity level greater than that predicted by adding

their individual toxicities (Preston et al., 2000). For example, lower concentrations of Fe can be toxic to *E. coli* in the presence of Al (Amador et al., 1999; Gutteridge et al., 1985), and Zn and Cu have been shown to act synergistically against *E. coli* (Preston et al., 2000).

Metal speciation determines the behavior of the elements and its reactions with organisms. It governs the element solubility and bioavailability (Reeder et al., 2006). The speciation of the metal cocktail showed that Al^{3+} is the dominant species at pH 4.0 (i.e., 86% of total Al). However, the $AlOH^{+2}$ proportion increased at MIC and MBC of the metal cocktail (Table 4.7) due to the increasing pH in the system (pH 4.5 at MIC and 4.7 and MBC). These results suggest that toxic species of Al include the Al^{+3} and $AlOH^{+2}$. The toxicity of Al^{3+} has long been recognized (e.g., MacDonald & Martin, 1988), and studies that include metal speciation at mildly acidic pH (5.0-6.0) also found that aqueous Al(OH) species are toxic (Amonette et al., 2003; Kinraide & Parker, 1989; Kochian et al., 1995). Mn and Zn, oxidation state +2, can also be antibacterial against enteric pathogens such as *E. coli* (Du et al., 2009; Faiz et al., 2011, Geslin et al., 2001; Harrison et al., 2005; Nies, 1999) at concentrations higher than measured in the AMZ experiments. Iron and Cu produce reactive oxygen species (ROS) via Fenton reaction series (Fenton, 1894) that are well known to oxidize cellular components (Imlay et al., 1988; Macomber et al., 2007; Morrison, 2016; Schoonen et al., 2006; Warnes et al., 2012).

Production of H_2O_2 is intermediate to production of ROS. The AMZ clay produced 0.4 μM over the course of 24 h, compared to the control kaolinite KGa-1, which did not produce measurable H_2O_2 (Figure 4.9). *E. coli* cells are affected by H_2O_2

concentrations greater than 25 μM; and ~ 500 μM is needed to achieve significant mortality (Hyslop et al., 1995). This suggests that production of ROS does not play a significant role the antibacterial process of AMZ. Another antibacterial clay from an epithermal sulfide deposit (Morrison et al., 2016) reported 50–500 μM production of H_2O_2 from dissolved metals. However, the membrane structure and function can be impaired by mechanisms not related to ROS. Here the evidence points to a significant role for Al.

NanoSIMS measurements confirm that the concentration of Al increased around the membrane of the *E. coli* that was reacted with AMZ (Figure 4.8, Table 4.8). Aluminum produces structural and functional changes in the *E. coli* outer membrane (Gutteridge et al., 1985; Guida et al., 1991; Oteiza & Verstraeten, 2006; Zatta, 2002). The outer membrane of *E. coli* was permeabilized after incubation with the AMZ, as shown by the vancomycin experiments. The breach in the membrane produced by the AMZ allowed the vancomycin molecule to penetrate and reach the antibiotic target (the peptidoglycan layer). Since high concentrations of Al were confirmed in the membrane by NanoSIMS analyses, it can be inferred that the Al damaged the outer membrane without production of significant H_2O_2. This suggests an attack of the membrane by a mechanism other than ROS degradation. Aluminum can alter the outer membrane by three possible mechanisms: binding to phosphate functional groups, displacing Mg, and promoting lipid peroxidation (Gutteridge et al., 1985; Guida et al., 1991; Oteiza & Verstraeten, 2006; Zatta, 2002). Aluminum has a strong preference for oxygen donors, which makes phosphate molecules, a major component of phospholipids, ideal targets for Al binding. Bound to phospholipids, Al alters protein interactions (Zambenedetti et al.,

1994; Garcidueñas et al., 1996), promotes aggregation of phosphorylated chains (Luque et al., 2014), and causes misfolding of proteins (Morrison et al., 2016).

Aluminum appears to play a central role in the antibacterial activity of the AMZ clay as it is the only element found at concentrations above the MIC for *E. coli* at pH 4.5. Aluminum increased in *E. coli* (Table 3.2, Londoño & Williams, 2016) and decreased in the AMZ clay (Table 4.4). However, it does not account for the total bactericidal effect observed. The combination of metals produced 100 % viability loss at the concentrations found in the LB incubated with AMZ. NanoSIMS isotopic mapping showed that relative amounts of Fe increased inside *E. coli* incubated with AMZ compared to the control. Aluminum primarily damages the bacterial membrane, and may potentiate oxidative damage produced by redox-active metals (i.e. those in the metal cocktail). Since the outer membrane of the cells is impaired, an unregulated influx of metals from the AMZ, could enter the cell and damage intracellular proteins, DNA, interfere with enzymatic activity, and alter Fe-S clusters (Chillappagari, et al., 2010; Macomber and & Imlay, 2009; Xu & Imlay, 2012).

Conclusion

This study investigated the role of metals in the antibacterial action of a lacustrine clay dominated by kaolins, in which Al toxicity appears to play a central role. It was shown that AMZ clay creates a mildly acidic environment that favors metal species that are toxic to bacteria. Aluminum targets the membrane by increasing its permeability, thus compromising the ability of *E. coli* to control the influx of metals. Understanding the antibacterial mechanism of AMZ has implications for its safe use as a natural

antibacterial product. The bioavailability of all elements in a natural clay must be considered, and weighed against its intermittent or persistent use.

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CHAPTER 5

PLACE-BASED ETHNOGEOLOGICAL CURRICULUM MATERIALS FOR TRIBAL SCHOOLS IN THE COLOMBIAN AMAZON

Abstract

This paper presents three place-based, culturally infused Earth-science curriculum units designed for use in tribal elementary schools (grades 4 and 5) in the Colombian Amazon. Development of the units was informed by sound principles of curriculum design, and of place-based and cross-cultural education. The three units teach basic Earth-systems principles and also integrate concepts from the indigenous ethnogeological knowledge of the Uitoto people of the Colombian Amazon. Although intended for a specific student population, they are offered as potential templates for Earth-science education in other cultural settings.

Introduction

"The territory is the basis of education"
(Muinane Elder, in Echeverri, 2008)

Similar to geologists, the indigenous people of the Amazon Basin study and interpret the natural features in their territory. Boulders, mountains, caves, palm swamps, rapids, and canyons, appear in stories as "enchanted" people, or as keepers, and as containers of either knowledge or disease (Bidigima & Silva, 1997; Echeverri, 2008; Urbina, 2010). Such meanings affixed to physical localities turn them into places (Tuan,

1977; Cresswell, 2014). The collective meanings and emotional attachments that people hold for a given place are referred to as the 'sense of place' (Basso, 1996; Hay, 1998; Stedman, 2003), a construct central to the practice of 'place-based education' (PBE; Apple et al., 2014; Gruenewald, 2003; Semken & Butler Freeman, 2008; Sobel, 2004; Smith, 2002; Woodhouse & Knapp, 2000). Sense of place is central to the cultural identities of indigenous peoples (Cajete, 2000; Kelley & Francis, 1994), and indigenous philosophies of education are intrinsically place–based, centered on traditional homelands (Semken & Morgan, 1997; Kawagley et al., 1998; Semken, 2005). Consequently, PBE is the most culturally authentic approach for teaching aboriginal or historically–resident students (e.g., Native American, Native Alaskan, and Hispanic/Latino students; Cajete, 2000; Riggs, 2005; Semken, 2005; Reano & Ridgway, 2015). There is also evidence that PBE makes geoscience studies more relevant and attractive to students in underrepresented minority populations (Levine et al., 2007). Educators who teach in a place-based manner, integrate culturally–relevant material as context in their lessons, and foster investigations of authentic science problems with their students, have been shown to be more successful in teaching Western science concepts and theories (Fensham, 2009).

Cross-cultural school settings call for teaching that is place-based, culturally–responsive and culturally relevant (Abrams et al., 2014; Ares, 2011, McKinley & Gan, 2014). Culturally–responsive schooling requires:

> "firm grounding in the heritage language and culture indigenous to a particular place (…) for the development of culturally-healthy students and communities associated with that place" (*Alaska Standards for Culturally–Responsive Schools*, Alaska Native Knowledge Network, 1998, p. 2)

Culturally-relevant education aims at "developing in learners an understanding of the world from different cultural worlds." (Mpfu et al., 2014). Thus, culturally–responsive and culturall– relevant schooling educates students to recognize, value, and apply their own cultural knowledge in a school setting while being able to appreciate, learn, and apply the knowledge of other cultures. These aspirations are shared by the tribes in the Colombian Amazon, as stated in their institutional educational projects and tribal government plans (AZICATCH, 2006; CRIMA, 2012; CICC, 1999). For example, the institutional education project of the school *Colegio indígena Casa del Conocimiento* (CICC) in La Chorrera, Amazonas, Colombia, advocates for an education that "strengthens the cultural identity of its members (history, language, and indigenous knowledge)". It also calls for culturally-relevant education that incorporates Western scientific views and helps students to be proficient at both cultural systems (CICC, 1999).

Earth science is taught at Colombian schools as part of a general natural-sciences curriculum. In La Chorrera, tribal members participated in a multicultural Biology degree, created by the Ministry of Education of Colombia (MEN) and sponsored by the National Pedagogic University, that aimed to develop cultural–content that could be used for tribal schools (e.g., Firizateke & Teteye, 2013; Giagrecudo-Dokoe, 2013; Giagrekudo et al., 2013). These works document traditional knowledge about plants, Earth origin myths, and the *maloca*, the place where knowledge is orally transmitted. These documents constitute valuable information that needs to be further adapted by natural sciences teachers to be included in curricular units. However, it is not clear how this content meets international or national education standards.

In this chapter, I present cross-cultural educational units that I designed for local Amazonian schools that tribal-school teachers in the Colombian Amazon can use to deliver Earth-science content in the Colombian Amazon. This material draws directly on the ethnogeological research presented in Chapters 1 and 2. The units are intended to increase interest in geoscience studies among indigenous students, foster cultural appreciation and environmental awareness, and share the outcomes of my research with indigenous communities in the Amazon.

Methods

Study Population and Setting

The Uitoto (also Huitoto, Witoto, Murui-Muina, or Muinane) are a native group who inhabit the northwest part of the Amazon Basin. Their traditional homelands are located at the interfluves of two main Amazon tributaries, the Caquetá and Putumayo Rivers, in Colombia (Pineda–Camacho, 1985). They are part of a larger cultural group that includes the Nonuya, Okaina, Bora, Miraña, and Andoke tribes.

The villages of La Chorrera and Araracuara have tribal educational institutions, specifically boarding schools and day schools, that encompass elementary, middle, and high school education. The schools serve mostly indigenous students, most of them Uitoto (80 % in La Chorrera; and 100 % in Araracuara; CICC, 1999; CRIMA, 2012).

Three units on Earth systems were designed for 4th and 5th grades, using place-based, culturally–responsive, and culturally–relevant education. Content for the lessons was drawn from my ethnogeologic research in the Colombian Amazon.

Educational Materials and Method

Three lesson plans were designed using the backward design method for curriculum development (Wiggins & McTighe, 2005), which comprises three steps:

1. Identify desired results (i.e., standards, goals, learning outcomes),

2. Determine acceptable evidence, and,

3. Plan learning experiences and instruction.

A backward-design template provided by Wiggins & McTighe (2005) was used for the design process (Figure 5.1.)

Desired results. Desired results are divided into four categories: enduring understandings, established goals, essential questions, and essential ideas. Established goals were taken from the U. S. *Next Generation Science Standards* (NGSS, 2013) and from the *Colombian Basic Competencies Standards for Science* (MEN, 2006). The specific goals were identified according to the learning experiences and instruction plan that the unit addressed. Enduring understandings were taken from the *Earth Science Literacy Principles* (ELSI, 2010). Essential questions and essential ideas were conceived according to the Next Generation Scienc Standards (NGSS, 2013) and learning experiences chosen from the internet.

<table>
<tr><th colspan="2">STAGE 1. DESIRED RESULTS</th></tr>
<tr><td rowspan="4">Established Goals
What relevant goals will this design address?
(e.g., content standards, course or program objectives, learning outcomes)</td><td>Enduring Understanding</td></tr>
<tr><td>What are the big ideas?</td></tr>
<tr><td>Essential Ideas</td></tr>
<tr><td>What specific understandings about the big ideas are desired?</td></tr>
<tr><td></td><td>Essential Questions</td></tr>
<tr><td></td><td>What provocative questions will foster inquiry, understanding, and transfer of learning?</td></tr>
<tr><th colspan="2">STAGE 2 - EVIDENCE</th></tr>
<tr><td colspan="2">Assessment Evidence</td></tr>
<tr><td colspan="2">Performance Expectations
Through what authentic performance task(s) will students demonstrate the desired understandings? (e.g., performance expectations NGSS, 2013)

Other Evidence
• Through what other evidence (e.g., quizzes, tests, academic prompts, observations, homework, journals, etc.) will students demonstrate achievement of the desired results?
• How will students reflect upon and self-assess their learning?</td></tr>
<tr><th colspan="2">STAGE 3 – LEARNING PLAN</th></tr>
<tr><td colspan="2">Learning Activities

Suggest activities that meet the standards and the criteria for sound ethnogeological education.</td></tr>
<tr><td colspan="2">References

List of works cited, activities or resources useful to teach the topics that the unit addresses.</td></tr>
</table>

*Figure 5.*1 Backward design template used to design the lessons. Modified from Wiggins & McTighe, 2003.

Evidence. Acceptable evidence constitutes the instruments that teachers use to document and validate that the students have achieved the desired results (Wiggins and McTighe, 2005). Performance expectations (NGSS, 2013) are performance tasks that the students should be able to do after completing the learning activities. Other assessments include informal checks for understanding (e.g., oral questions); tests and quizzes; academic prompts (e.g., open ended questions or projects; Wiggins & McTighe, 2005; 2011). The teacher may choose to assess content knowledge acquisition; skills; changes in attitudes, values, or beliefs; or long-term behavioral outcomes (Libarkin & Kurdziel, 2001).

Learning plan. The learning plan includes learning activities and references. Learning activities are instructions designed to help teachers deliver the unit. The teacher can use or modify the suggested activities. The plans presented here were modified from lessons available online to include place-based education, culturally–responsive and culturally–relevant schooling. The activities correspond to the standards that the units address and were modified to emphasize the regional or local environment and to include traditional knowledge when available. Lastly, the references include cited works and links to resources. The learning activities include experiential learning activities such as games, demonstrations, field work (Riggs, 2005) and using cultural stories to makes sense of geological events (Abrams et al., 2015).

Results

As a proof of concept, three sample units were designed: *Earth systems in northern South America* (4th grade), *water distribution in the planet* (5th grade), and *Earth*

systems: What processes affect our planet? (Earth systems, 5th grade). The sample units are presented in Appendix F.

The unit on *Earth systems in northern South America* is focused on the origin of the Amazon River and Amazon rainforest. It is designed for 4th grade. The teacher starts by asking students to observe a Google Earth © image of northern South America, then guides the students to identify the main geographical features (i.e., Amazon River, Andes Mountains, Atlantic and Pacific oceans) by leading them with questions. Once the students observe the distribution of features at a continental scale, they are challenged to think about reasons for such a distribution. Ethnogeologic content is introduced with the Uitoto myth that narrates the formation of the Amazon, referred to as the *Moníya aména* story (Chapter 2; Londoño et al., 2016). Storytelling is an essential part of Uitoto education (Bidïgïma & Marin, 1997; Micarelli, 2015); this narrative engages their cultural identity in the learning process serves as a framework to which the students can attach Western knowledge. Further, many of these stories can contain significant observations and patterns of natural events that could be used in education (Abrams et al. 2014: Ault, 2008; Hudicourt-Barnes, 2003; Johnson et al., 2014; Londoño et al., 2016).

To understand the Western geoscientific model for the formation of the Amazon, students require a general understanding of geological processes, such as weathering, erosion and deposition, and plate tectonics that result in mountain uplift. Therefore, the unit includes two experiential activities that cover weathering, erosion and deposition, and plate tectonics. Following these activities, the teacher can assess the students' grasp of the background knowledge. If this is deemed sufficient, instruction proceeds with

Western geoscientific model for the formation of the Amazon River (Hoorn, 2006). This is presented in a narrative format similar to that of the Uitoto ethnogeological content, but it also draws on data. The teacher makes explicit the parallels between the Western and traditional story to engage previous knowledge and promote cultural awareness. In the last part of the unit, the teacher helps the students articulate the knowledge seen in the unit with the final activity, which also serves as a cumulative assessment.

The unit titled *Earth Systems: What processes affect our Planet?* is designed for 5th grade. It addresses the interactions among the four spheres of the Earth system: geosphere, hydrosphere, atmosphere and biosphere (e.g., ESLI, 2010). Understanding these concepts requires that students think about systems, and acquire the language typically used to explain them. The teacher checks for prior knowledge by asking students about systems they know of. Activating prior knowledge helps students articulate new content and use it more effectively (Ambrose et al., 2010). The unit continues with the lesson and worksheet for students 'thinking about systems' guide, adapted from AGI (2013). The guide was adapted to the cultural context, using examples of systems that make sense to the Uitoto, such as a traditional drum (*maguaré*) and the system of traditional dances (*bailes*), the human body, rivers, ant hills, etc. It also includes indigenous knowledge about the three main natural spheres for the Uitoto (the world below, this world, and the world below). The similarities and differences between the Uitoto and the Western Earth system spheres should be made explicit by the teacher to avoid misconceptions. The unit ends with a brief active exploration conducted just outside the classroom (adapted from AGI, 2013) that allows students to interact with their local environment and apply their understanding of systems to the real world.

The distribution of water in the planet is the topic of the third unit. Water is essentially the most abundant resource in the territory of the Uitoto, and hence it may be difficult for Uitoto students to comprehend that relatively little of Earth's water is found in rivers or elsewhere on the continents. By means of a demonstration performed by the teacher (Water, water everywhere), the students are introduced to the relative distribution of water through the Earth's hydrosphere. The discharge of the Amazon River is included in the last step of the activity to make an important local connection. The students will understand that, although the Amazon region is hydrologically–rich, surface water is very limited, and clean water is precious to humans.

Future Work

Currently, the three units are limited to proof of concept models that have not been tested in actual tribal-school classrooms. Before this can be done the lessons must be reviewed and possibly modified (Ward et al., 2014) by indigenous collaborators, then translated from English into Spanish and Uitoto, and finally reviewed by the teachers in the local tribal schools before implementation.

It is suggested that the effectiveness of the units can be tested by a quasi-experiment in which they are implemented in an experimental class section, while the existing curriculum is delivered to a control class section. Learning outcomes in content knowledge, cultural knowledge, and sense of place can be tested pre- and post-course by means of a number of validated, published instruments and methods from the geoscience-education literature (e.g., Semken & Butler Freeman, 2008; Williams & Semken, 2011; Ward et al., 2014).

Conclusions

This chapter illustrates how three curriculum units were conceived and an designed to deliver cross-cultural Earth-science lessons to elementary students in tribal schools in the Colombian Amazon. The curriculum is situated in the local environment, focused on landscapes and resources of local importance, and integrates ethnogeologic knowledge obtained through research conducted in collaboration with indigenous Uitoto. These units are offered as an initial step towards applying ethnogeology to Earth-science education in the Colombian Amazon.

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The Colombian Amazon is the traditional home of ~50 % of the Colombian ethnic groups, and it contains the largest indigenous reservations in Colombia (National Planning Department, 1997; Dane, 2012). Environmental knowledge and taxonomy of water and the land were explored in this study using methods from ethnosciences. Because the indigenous models are holistic, geology content is found within a broader ecological and cultural context. This dissertation shows that the Uitoto people, indigenous to the Colombian Amazon, have ecological models that explain the evolution of the Amazon, not only as a river, or river network, but as an ecosystem. Similarly, local water taxonomy includes ecological criteria. The different water categories explored in Chapter 2 were explained in terms of differences of the major element composition, providing a scientific basis for such differences.

The Uitoto exploit the medicinal properties of clay minerals found in their territory; one of these clays is the AMZ clay. Using Western Science methods, this study concludes that the antibacterial mechanism exhibited by the AMZ clay is primarily a result of aluminum toxicity, and is different from other clays in that it does not rely on reactive oxygen species, or on large concentrations of Fe. The aluminum accumulates in the bacterial membrane and increases its permeability. The low concentrations of transition metals sourced by the AMZ clay (below their individual minimum inhibitory

concentrations) appear to act synergistically to enhance the chemical attack on bacteria. Medicinal or therapeutic uses of clay need to address secondary effects of aluminum and transition metals.

Ethnogeology contributes specific cultural information about geological processes and mineral resources, but also how the people have historically adapted or dealt with such processes, and how mineral resources are exploited by the biota, in other words, ethnogeologic studies have the potential to contribute to Earth System science and to human sciences. One way in which research results can be shared and returned to the indigenous peoples is in the form of educational materials (e.g., curriculum, lesson plans, learning activities). This work shows examples of cross-cultural materials of Earth science systems. The materials can be used by teachers in the local schools of the Colombian Amazon.

Future Work

Geology of the Amazon

The surface geology of the Colombian Amazon warrants further study. Recent versions of the official geological map (e.g., Gómez et al., 2010) still recreate the mapping performed with images from the radar-grametric project (PRORADAM) conducted in the 70's (Galvis et al., 1979). Further work should consider: 1. producing a more detail geologic map of the Araracuara and La Chorrera region (scale 1:100,000 or 1:50,0000); and determining the extent of the antibacterial clay deposit.

In this work, an attempt was made to determine the age of the antibacterial sample (using palynology, Appendix D.14). However, the sample sent to the lab did not contain pollen. Future work could consider a field campaign to collect samples that can be dated, either by pollen, or by cosmogenic isotopes. The geologic history of the antibacterial clay deposit is uncertain. The deposit was assumed to pertain to the Pebas formation based on cartography and literature, however, this needs to be confirmed. The source of the sedimentary grains inherited by the AMZ clay is unknown. Although the proximity to the Andes, and the current drainage direction could indicate that the heavy and transition metals were sourced by the cordillera, it is possible that the Guyana shield acted as a source if: 1. The Guyana shield in the Araracuara and Chiribiquete ranges sourced the minerals (Figure 1.1), or 2. if the sedimentary deposit is older than the uplift of the Northern Andes, then most likely the source of the sediments is the Guyana shield, at the East.

Ethnogeology

Further work could explore in more detail the classification of wetlands, using cultural consensus analysis, participatory field trips and structured interviews. Significant biogeochemical connections could be explored from the cultural knowledge and Western science, for example: How does the *Mauritia Flexuosa* palm influence the water in palm swamps? Or how does the distribution of fish change in different types of hydraulically isolated water bodies?

The study of cultural knowledge of geological resources can be extended to include the classification of landscape features and rocks found in the Colombian

Amazon and their origin, according to the Uitoto. This would record the indigenous knowledge and serve as a bank of information from which educational materials could be designed.

Antibacterial clay research

A mixture of clay minerals forms the AMZ clay (disordered kaolinite, halloysite and smectite). Future work could address the question: What is the contribution of each mineral to the antibacterial effect? Since the constituent clay minerals in the sample cannot be easily separated, reference clay standards could be used as a proxy. Experiments with each mineral would include: Which mineral contributes more structural metals (e.g., Al) at 37 °C during 24 h? And is the concentration found in the media (water, isotonic solution, and LB) inhibitory or bactericidal? The dissolution could also be assessed using Electron microscopy and ICP-MS, determining the composition of minor and trace metals before and after the experiment. An interesting variation would be to study the contribution of organic acids or molecular components, by-products of bacterial metabolism, in the process of dissolution. It is possible that such molecules could catalyze the dissolution process. To do that, a high biomass of *E. coli* would be cultured in media overnight. Then the cells would be pelleted and the supernatant could be incubated with clay, to determine if dissolution was enhanced.

To increase our knowledge about antibacterial clays, a database with chemical data for all the antibacterial clays identified could be constructed. Then, statistical analysis could help us identify patterns or components common to all antibacterial clays. The data base would contain chemical data (e.g., ICP-MS of major, minor, and trace

elements) for the leachate, exchange solution, and clay. Then analysis such as boxplots, or Whisker plots, explained in appendix D1, could be performed. Other important information to be included in the data base would be geological setting of the sample, organisms that have been tested, and any cultural information available.

Science Education

In this study, I designed 3 sample lessons. Future work should include revisions by the community, implementation in the classroom and assessment. A instrument, e.g., a survey, will be designed to measure increased sense of place following the methods of Semken et al. (2009) to measure sense of place as a learning outcome. The cycle of design, implementation and assessment can be repeated with new data collected from ethnogeologic studies.

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APPENDIX A

HUMAN SUBJECTS RESEARCH EXEMPTION

INSTITUTIONAL REVIEW BOARD

ASU Knowledge Enterprise Development

Office of Research Integrity and Assurance

To:	Elizabeth Brandt MC
From:	Mark Roosa, Chair Soc Beh IRB
Date:	11/29/2012
Committee Action:	**Exemption Granted**
IRB Action Date:	11/29/2012
IRB Protocol #:	1211008533
Study Title:	Ethnosciences and Geology. A research avenue for Geosciences in Colombia. Phase I. Fluvial Systems in NW Amazonia

The above-referenced protocol is considered exempt after review by the Institutional Review Board pursuant to Federal regulations, 45 CFR Part 46.101(b)(2) .

This part of the federal regulations requires that the information be recorded by investigators in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects. It is necessary that the information obtained not be such that if disclosed outside the research, it could reasonably place the subjects at risk of criminal or civil liability, or be damaging to the subjects' financial standing, employability, or reputation.

You should retain a copy of this letter for your records.

APPENDIX B

PUBLICATION CITATIONS

Chapter 2 titled "ETHNOGEOLOGY IN AMAZONIA: SURFACE-WATER SYSTEMS IN THE COLOMBIAN AMAZON, FROM PERSPECTIVES OF UITOTO TRADITIONAL KNOWLEDGE AND MAINSTREAM HYDROLOGY " is reprinted in this thesis with permission from co-authors: Cristina Garzón, Steven Semken, Betsy Brandt, & Vicente Makuritofe[4]. The original article was published in 2016 in G. R. Wessel & J. K. Greenberg (Eds.), *Geoscience for the Public Good and Global Development,* Special Paper of the Geological Society of America*,* volume 520, pages 221-232.

Chapter 3 titled " UNRAVELING THE ANTIBACTERIAL MODE OF ACTION OF A CLAY FROM THE COLOMBIAN AMAZON" is reprinted in this thesis with permission from co-author Lynda B. Williams. The original article was published in 2016 in *Environmental Geochemistry and Health,* volume 38, issue 2, pages 363-379.

[4] Makuritofe V. deceased

APPENDIX C

ERRATA

Ion chromatography data appeared for specie NO_3^- in Table 2.2. A cautionary note appeared in Table 2.2 regarding this, and other species. The data is not reliable due to sampling limitations and it should not be used or cited.

Figure 2.6. The figure in Chapter 2 has been replaced. The figure published (Figure C.1) contained the vector for fluor (F), an element that is below detection limits. The axes were labelled erroneously as Comp. 1 and Comp. 2. In the Figure 2.6, Chapter 2, the labels were corrected as PC1 and PC2 and the F has been removed. The caption of C.1 is the corrected caption for Figure 2.6

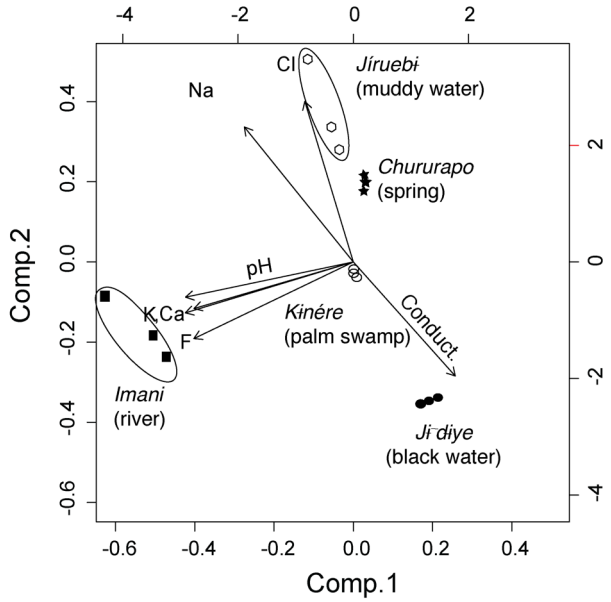

Figure C.1 Biplot of the principal component analysis of 15 water samples. The left and bottom axes show normalized principal component scores, the top and right axes show loadings.

APPENDIX D

EXPERIMENTS

Experiments to Characterize Clays and its Derivatives				
#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.1.	**Q**. What elements do antibacterial clays (ABCs) have in common? **H.** Antibacterial clays have elements in common that differentiate them from non-antibacterial clays (non-ABCs).	To compare the chemistry of the leachates, exchange solutions, and digested clays of antibacterial clays (OMT, Walker, Ag02, and AMZ) and non–antibacterial clays (Kao API #5, Arg02, and OMT red) boxplots were employed. To create them, each concentration for each element of ABCs and non-ABCs was standardized so all the concentration values could fit on one plot. To compare the two groups: ABCs and non-ABCs, I used the non-parametric rank sum test. This test ranks the concentrations of the clays for each element with 1 being the smallest. The sum of the ranks for each group (ABCs and non-ABCs) is then compared, and a p-value is produced. This test was performed with the assistance of the statistics consulting group, at ASU.	For digested clays, the elements that were significantly different were: B, V, Cu, Ga, Ta (p-value<0.05). For the leachates, Li, Si, Cu, As and U (p-value < 0.10). No significant comparisons for the exchange data. These particular elements may just need a larger sample size to prove significance and may warrant further testing.	**Q.** Answered **H.** Tested and confirmed but the results are preliminary.
D.2. XRD	**Q.** What is the mineralogy of the AMZ? **H.** The AMZ sample is mostly composed of clay minerals.	X-ray diffraction (CuKα radiation, Bruker D 5000). Samples were prepared for random-powder analysis using alumina (10%) as an internal reference mineral. The quantitative mineralogy was performed using RockJock. The detailed clay mineralogy was modeled using Newmod (Reynolds, 1985).	The AMZ clay is a mixture of clay minerals: kaolinites 45 %, smectite 36 %, quartz 15 % (Figures 3.1 and 4.2) Newmod results: Disordered kaolinite (R0), di-mica, Illite R1 Mixed-layer clays: smetite/illite (80% smectites), Illite/smectites (80% Illite), kaolinite/smectites (80% kaolinite).	**Q.** Answered. **H.** Confirmed.
D.3 HI	**G.** Characterize the defects present in the kaolinite of the AMZ. **H.** The kaolinite in the AMZ is disordered, the HI will be low.	Measure the Hinckley Index (HI) in the XRD pattern.	HI of the AMZ = 0.8, indicates moderately disordered. It could also been interpreted as two types of kaolinites: one more disordered than the other one.	**G.** Achieved. **H.** Confirmed

#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.4. Major Elements	**Q.** What is the major element composition of the AMZ? **H.** Major elements will be dominated by Al and Si.	Determined in two ways: X-ray Fluorescence and electron microprobe. XRF: Samples were pelleted (hydraulic press) and analyzed in a MagixPro PW-2440 Phillips at the National University of Colombia. Another sample was analyzed at the USGS, Denver, CO. Elements analyzed: SiO_2, Al_2O_3, K_2O, Fe_2O_3 (total Fe), TiO_2, Na_2O, MgO, P_2O_5, CaO, and MnO. Electron microprobe (JEOL JXA-8600, ASU): samples were powdered, mounted on carbon slides, and C- coated. 15 kV beam, 10 µm probe diameter. The elements analyzed were: SiO_2, Al_2O_3, K_2O, FeO (total Fe), TiO_2, Na_2O, MgO, and CaO.	Results for all three analyses, using different methods, are similar for the AMZ clay (Table 3.2). The AMZ clay has expected composition for clay minerals (mostly composed of SiO_2 and Al_2O_3).	**H.** Confirmed
D.5. Minor Elements	**Q.** What is the minor and trace element composition of the sample?	100 mg of AMZ clay and reference kaolinite (Kao API#5) were digested by hot plate digestion in concentrated acids of AMZ clay (EPA method 200.8, Brockhoff et al., 1999). The samples were analyzed by ICP-MS in 2 % HNO_3 matrix.	The AMZ contains a variety of transition metals, some of which could be antibacterial (Table 3-2). Results need to be compared to MIC and MBC at pH 4.5.	**Q.** Answered. It is necessary to determine which of the transition metals is exchangeable or soluble in water.
D.6. TSSA	**Q.** What is the Total surface area (TSSA) (i.e. the maximum area accessible to water, exchangeable cations, and polar molecules?) **H.** The AMZ clay has a surface area that exceeds that of kaolinites.	Samples dehydrated overnight at 120 °C. 1 g of clay saturated with 3 mL EGME (Carter et al., 1986). SWy-1 was included as a control. Samples were placed inside a desiccator containing $CaCl_2$ and EGME, and the excess EGME was removed under vacuum. The weigh of the samples was monitored after 18 and 24 h. The total surface area was estimated using the equation: $$TSSA = \frac{W_{EGME}}{2.86 \times 10^{-4}} \times Ws$$ where W_{EGME}: weight of EGME (g) retained by the sample, Ws: oven-dry weight (g) of soil used, and 2.86×10^{-4} g: the calculated quantity of EGME required to cover 1 m^2 of clay surface with an EGME monolayer (Cerato & Lutenegger, 2002).	TSSA (AMZ) is 5 times ≥ TSSA (KGa-1), and it is ≤ ½ TSSA area (SWy-1) The fraction of smectites present in the AMZ samples increases the surface area when compared to kaolinites.	**Q.** Answered. **H.** Confirmed.

#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.7 SSA	**Q.** What is the external surface area of the AMZ and how does it compare to reference clays?	The samples were dried at 110 °C overnight in a stream of N_2. The samples were outgassed (130 °C for 6 h) under a vacuum of 0.1 mm Hg and then conditioned with N_2 (flowing stream) at 35 °C for 20 h. N_2 adsorption was determined for every sample (AMZ, and other known or suspected antibacterial clays) over the relative equilibrium adsorption pressure (P/Po) range of 0.05-0.25. The BET surface area was analyzed in a TriStar II 3020 (Micrometrics), managed by the CLAS Goldwater Environmental Laboratory at ASU.	AMZ's specific surface area is greater than that of the reference clays (KGa-1 and SWy-1)	**Q.** Answered.
D.8. EM-SEM	**G.** Observe the morphology of the AMZ clay and its surface.	Samples of AMZ clay and KGA API#% was C-coated and observed using Philips XL 30 FE-SEM.	AMZ's surface has etched pits, is rough, heterogeneous and may have precipitates of oxides.	**Q.** Answered.
D.9. CEC	**G.** Determine the CEC of the AMZ and compare it to standard clays	0.7 g dry weight of AMZ were mixed with 25 mL of a 0.04 N Co(III) hexamine chloride solution, sonified for 2 min, and shaken for 1 h in a hand-wrist shaker. Clays were separated by high speed centrifugation and 5 mL of the supernatant were transferred into plastic tubes. The absorbance at 472 nm was determined for the supernatant and for the 0.04 N solution in a UV/VIS spectrophotometer. The assay was performed in duplicate. CEC was calculated using the equation: $$CEC_{A472} = \left[\frac{A472_{0.04N} - 472_{assay}}{A472_{0.04N}}\right] \times 44 \times \frac{V}{m} \times 100$$ where: A_{472}: absorbance of the Co-hex solution and the assay, 44: normality of solution expressed as meq/L, *V:* volume in L of the Co-hex solution mixed with *x* g of dry clay. (i.e., 0.025 and 0.7 respectively). A reference clay (SWy-1) was included to assess the accuracy of the test (Ciesielski et al., 1997; Aran et al., 2008).	The CEC of AMZ is in between the values of the reference kaolinite (KGa-1) and the reference smectite (SWy-1) due to its smectitic composition. CEC is important because transition metals that can be toxic and could be hosted in the interlayer. I need to find out what is the composition of the exchangeable fraction.	**Q.** Answered.

#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.10 Exchanged cations	**Q.** What cations can the AMZ exchange and in what concentrations? Are there transition metals in high concentrations that could be toxic to *E. coli*?	Two compounds were used: NH_4OAc and NaCl, using different clay concentrations. 1. The AMZ clay was shaken with 1M NH_4OAc (10 mg clay/ mL exchange solution) for 24 h, the exchange solutions analyzed by ICP-MS and analyzed in a 2% HNO_3 matrix. 2. The AMZ clay was exchanged with 1M NaCl (100 mg clay/ mL) and analyzed in a 2% HNO_3 matrix.	1. The elements in the exchange solutions prepared with NH_4 acetate (Table 3.5). Elements are below MICs reported in the literature, the data obtained with NH_4OAc does not support a chemical mode of action. However, this experiment was problematic because NH_4Ac does not dissociate completely and because the clay concentration was too low. 2. Extraction with 1M NaCl (Table 4.3) was made with a preferred cation and using a greater clay: liquid ratio (above the MIC of the clay.) I found higher levels of Al and Cu.	**Q.** Answered.
D.11. Leachtate	**Q.** What water–soluble elements are released from the clay after 24 h of equilibation?	The leachate was prepared in two different concentrations: 50 mg clay/mL (from Williams et al., 2008), and in 250 mg/mL, which is the MBC of the AMZ when using 5 g/L LB.	The concentration of the chemical elements is reported in table 4.3 at the 50 mg clay/mL concentration. The results of the concentrated leachate are not shown because only one sample was analyzed. However, no element is above the MIC in the more concentrated leachate. The concentration of the elements is low compared to the leachates of other antibacterial clays.	**Q**. answered.
D.12. LB	**Q.** What is the chemical composition of the Lysogeny broth?	The LB was analyzed at a concentration of 5 g/L and 25 g/L. Samples were analyzed by ICP-MS	LB broth has essential and micronutrients. Results are reported in Table 4.3. This data is useful to compare the change in the LB when incubated with AMZ.	**Q**. Answered.

#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.13. pH & Eh	**G.** Assess the pH and ORP in the poultice during overnight incubation, in a ratio that simulates a poultice. **H.** The known antibacterial clays (French green clay and OMT) buffer the pH of a solution to either acid or basic conditions. My hypothesis is that the water in contact with AMZ clay will be acid or basic after 24 h of mixing with the clay.	2 g of clay were mixed with 8 mL DIW (250 mg /mL). The pH and Eh (ORP, RmV) was continuously recorded with a Thermo Scientific ORION Dual start meter. The instrument recorded the data automatically. The instrument was calibrated with Zobell solution.	At room temperature the pH of the AMZ decreased from 4.8 and 4.3 in the mixture. The Eh varied between 361 and 361.7 mV. For the kaolinite pH decreased from 5.9 to 5.5 and the Eh from 368 to 331 mV.	**G.** Achieved. **H.** Confirmed, the AMZ clay generates acidic conditions, different from the control kaolinite (Kao API#5). The Eh is comparable between the two.
D.14. Palynology	**Q.** Is the AMZ sample from the Pebas Fm.? **H.** Based on stratigraphic correlations, the AMZ sample comes from the Pebas Formation (Miocene).	Pollen analysis of a sample (performed at the labs of the University of Amsterdam, thanks to Carina Hoorn	No pollen recovery. The geologic map of Colombia uses different names of Formations. The geology of the Colombian Amazon needs further study and mapping.	**Q.** Not answered

Basic Experiments in Microbiology				
#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.15. Cal curve Spec. OD600 vs CFU/mL	**G.** Generate a calibration curve for the spectrophotometer. Determine the correspondence betweenOD600 and Colony Forming Units/mL for *E. coli* (ATCC 25922) and *B. subtilis* (ATCC 6633) (this curve is a function of the microbe and the spectrophotometer)	*E. coli* was grown overnight and a fresh culture was started in the morning. The bacterial rate of growth was monitored by measuring the culture OD600 every hour and after overnight incubation. Parallel to this, 100 µL of the experiment were serially diluted, plated in LB agar and incubated overnight. Colonies were counted on the plates and the number of cells in the original suspensions were calculated by dividing the number of colonies by the product of the volume plated and the dilution factor. A curve OD600 vs CFU/mL was constructed. The experiment was performed in triplicate.	With more diluted LB, the optical density of the culture changes. In 25 g/L LB, the OD600 between 0.4-0.5 corresponds to 10^8 CFU/mL. In 5 g/L LB the same concentration corresponds to an OD600 between 0.2 and 0.3. The values were similar for *E. coli* and *B. subtilis*, and also between spectrophotometers. These sets of experiments produced a growth curve, or a microbial growth rate curve of cell density vs. time, in which the lag-phase, exponential growth phase and stationary phase for the microbes were determined. The OD600 vs. CFU/mL was key to make sure that the starting concentration of cultures (CFU/mL) was what I intended, thus it was a way to standardize the tests.	**G.** Achieved.
D.16. Viability Test	**G.** Quantify antimicrobial activity of AMZ in two model bacteria representative of Gram-positive (*B. subtilis)* and Gram-negative (*E. coli)* groups.	Sub-cultures of were grown to log phase. Cultures were diluted to a concentration of 10^7– 10^8 CFU/mL. Clays (previously weighed and autoclaved) were mixed with 1 mL culture and incubated overnight at 37 °C. The assays were serially diluted in isotonic solution (0.85 % NaCl) and plated on LB agar to determine viable colonies. Colonies were counted. Susceptibility to the clay was assessed by compared the cell density in the samples incubated with clay vs. the samples incubated without clay (bacteria control). Microorganisms tested: *E. coli, B. subtilis, S. epidermidis.*	This is the general form of the experiment. Things that were varied according to the purpose of subsequent experiments include the media (concentration, pH, type), the clay (sample and mass), and the starting volume of the culture. In the following experiments I will specify the media, clay used but the reader should refer to the general procedure for the basic guidelines	**Q.** Answered
D.17 Inhibition Ring	**G.** Test the antibacterial activity of clay using the inhibition–ring method.	In an LB-agar plate, spread a thin layer of bacteria. Punch holes on the plate and fill them with hydrated clay minerals. Incubate at 37 C, overnight. Measure inhibition ring the next day.	No inhibition ring formed around the wells. Could be because the antibacterial agent does not difuse in the agar, or because the mode of action of the clay is physical.	**Q.** Not answered by this method.

#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.18. Leachate	**Q.** Is the leachate antibacterial? **H.** If the leachate is antibacterial, the mode of action of the AMZ clay relates to a soluble specie that is leached above MIC.	Leachate tested by inhibition ring method, and by viability counts. Briefly, 1 mL of *E. coli* in log phase was mixed with 1 mL leachate (prepared by leaching 250 mg clay/mL in DI water).	Leachate is not antibacterial. Need to study leachate composition by ICP-MS.	**H.** Disproved.
D.19 Growth curve	**Q.** What is the rate of microbial growth in 5 g/L LB and 25 g/L LB? **G.** Characterize the growth of the microorganisms (*E. coli* & *B. subtilis),* determine the generation time and the parts of the growth curve in different media.	*E. coli* was inoculated in fresh LB, growth was monitored via optical density readings at 600 nm and plating.	Generation time of *E. coli* is 20 min in 25 g/L LB and 30 min in 5 g/L LB. For *Bacillus subtilis*, it is 28 m in in 25 g/L. The growth curve at 5 g/L LB was not determined for *B. subtilis*.	**Q.** Answered
D.20 Kill curve	**Q.** What is the rate of microbial death in isotonic solution and in 5 g/L LB	Incubate bacteria with 70 mg clay. Take aliquots of the suspension every 2, 6 and 20h. Do viability counts. Incubate a control of E. coli without clay. Performed in isotonic solution and in 5 g/L LB but the initial concentration of bacteria differ so they are not comparable.	Killing occurs in the first 8 h	**Q.** Answered
D.21 MIC / MBC (AMZ clay)	**Q.** What is the minimum dose of the AMZ needed to inhibit *E. coli* growth by half compared to the control (MIC)? What is the minimum dose of the AMZ required to to eliminate *E. coli* (MBC)? And, how does this change in different media (i.e. 5 g/L LB, 25 g/L LB and isotonic solution; 0.85 % NaCl).	The general viability count test was used but using the following concentrations in (mg/mL): 25 g/L: 100, 250, 300, 350 5 g/L LB: 50, 75, 100, 200, 250, 275 0.85 % NaCl: 60, 90 mg/mL	The amount of clay needed to inhibit or eliminate bacterial growth varies according to the media, the MIC and MBC are increase in the following order: isotonic solution > 5 g/L LB > 25 g/L LB. Results are reported in Table 4.2. The buffering capacity of the media, and the chemical composition, which can react with metals, may be responsible for this effect.	**Q.** Answered. The data from this experiment is useful in the design of future experiments (choosing a dose to obtain certain results).

#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.22 AMZ in DIW	**Q**. Is the AMZ clay antibacterial in water? Or is it only antibacterial in LB?	Fresh cultures of *E. coli* were grown to exponential log phase in 5 g/L LB. Aliquots of the culture (1 mL) were pipetted into Eppendorf tubes and the LB media was washed with 0.85% NaCl. Cells were pelleted and re-suspended in de-ionized water. 70 mg/mL clay were added to three of the Eppendorf tubes, the other three were incubated as controls. The pH and Eh were measured after 24 h.	The *E. coli* incubated with AMZ lost 100 % viability The ~~control~~ *E. coli* grow less than it usually does in other media (isotonic or LB), its population decreased in an order of magnitude. The DI water does not have any nutrients and produces osmotic shock, reducing *E. coli's* viability. Since the idea in my experiments is to observe the effects of the AMZ clay, and not the ones of water, the media has to be replaced with isotonic or complex media (0.85% NaCl).	**Q.** Answered. The AMZ is antibacterial in water. This is a good result for applications of a clay poultice. However, it is not a good media to use in my experiments.
D.23 Cell Separation	**G.** Retrieve *E. coli* reacted with AMZ for further analyses.	Two different methods were used: a procedure developed to image clays and bacteria via Transmission Electron Microscopy (TEM), and, a procedure modified from that uses Nycodenz, used for ICP-MS analysis and NanoSIMS. Both methods were modified from Neveu et al. (2014) and Tovar-Sanches et al. (2013). Both methods described in Chapter 3.	These two methods helped to separate bacteria from the bulk of the sediments, but the bacteria recovered was not free of sediments. The advantage of the first method is that it does not require Nycodenz, which is expensive. The advantage of the second method is that it provides more biomass, needed for ICP-MS analysis.	**G.** Achieved.
Experiments in which the Clay was Altered to Identify Characteristics Related to the Antibacterial Mode of Action				
D.24 Size	**Q**. Does the antibacterial property depend on the size fraction? **H.** The finer fraction is antibacterial	Separate the [2.0-1.0], [1.0-0.5], [0.5-0.2] and < 0.2 µm fractions of the AMZ by centrifugation. Perform a viability test with 250 mg of each clay fraction against *E. coli*. LB used was 25 g/L. (Clay dose = MIC 25 g/L LB)	The different size fractions reduced *E. coli* viability within error. No significant differences were detected ($p>0.1$). Thus, whatever is responsible for the killing is present and operating throughout the size range. The antibacterial agent(s) or process is present, or works in all different size fractions.	**Q.** Answered.
D.25 Exchanged	**Q**. Does the exchanged clay maintain its antibacterial properties? **H.** If the mechanism of the AMZ is entirely physical, the AMZ clay will remain bactericidal after the cations have been exchanged.	Mix clay with 1 M KCl (10 mg/mL mineral/liquid ratio), shake for 24 h in hand-wrist action shaker. Centrifuge samples at high speed. Wash excess Cl from clays with nanopure water. Dry and autoclave clays. Conduct a viability test as outlined in the viability count procedure	After cation exchange, the clay is no longer antibacterial. Results indicate that the antibacterial effect depends on either exchangeable species or processes facilitated by surface charge	**Q**. Answered. **H** Disproved. Yet, is possible that the antibacterial effect is related to the electric charges of clay minerals.

#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.26 DCM MEOH	**G.** Rule out that the antibacterial mode of action is due to organic compounds present in the clay. **H**. The mode of action is not related to organics, therefore the clay will retain its antibacterial property.	Organics extraction: Mixed 1 g of AMZ with 5 mL DCM and 5 mL MEOH, left to soak overnight, treatment repeated three times. The clay was dried and sterilized previous to testing via viability count. Two concentrations of clay were tested: 70 (sub-lethal) and 100 mg/mL and compared to AMZ samples that were not treated with DCM/MEOH.	No significant difference (p=0.2) between the AMZ treated with DCM/MEOH and the AMZ without treatment.	**H.** Confirmed, the antibacterial effect is not due to organics extractable with DCM/MEOH. It is also not due to volatile organics that were extracted during autoclaving processes.
Experiments to Test if the AMZ's Mode of Action is due to Physical Interactions				
D.27. EM-TEM	**G**. To observe the appearance of cells, observe mineral and bacteria interactions. H. If the mode of action is physical then cell membranes will be disrupted, pierced, or minerals will be around cells suffocating them.	Incubate for 24 h *E. coli* and *B. subtilis* with AMZ (80 mg clay/mL culture) and KAO API#5 as a control. Separate the bulk of minerals from the cells. Do TEM mounts in Cu grids using chemical point drying. Stain the grids to increase contras. A time series was developed and 'snapshots' of the process were taken after 2, 4, 6 and 8 h) t	*E coli* and *B. subtilis* cells were observed with TEM after incubation with clay and without clay (Fig. 3.5). Nanoparticles of minerals were seen attached to the cell wall, but in very low density. Clays were identified by their hexagonal shape, some of them in edge-edge contact with membranes. Anomalies in the membrane: differences in texture and morphology, black particles inside the outer membrane and membrane detachment were observed. The images compared to results with the OMT differ. Visually it seems to be a different mode of action. The cell density in TEM mounts was too low to have enough counts. However, the nanoparticles and membrane anomalies may be useful information.	**G.** Achieved. **H**. Not testable by observation only, the images are interpretative and results could be biased due to lack of statistics. Data needs to be compared, contrasted and confirmed using other techniques.
D.28. ζ_{pot} and DLVO	**G**. Calculate the electrostatic interaction, and potential for attraction, between clay (AMZ, KAO API#5) and bacteria (*E. coli & B. subtilis)*. **H**. The energetic barriers between clays and particles will be low between bacterial cells and clay, that is, there will be forces of attraction.	Suspend clay in its leachate (1 mg/mL). Measure Zeta potential (mV) in ZetaPALS: (Brookheaven Instruments) over the pH range between 1-9. Then apply DLVO theory to find the energetic barriers between a cell and clay minerals at pH 4.5 (de Kerchove & Elimelech, 2005; Gregory, 1981; Hogg et al., 1996; Redman et al., 2004).	Both surfaces (clay and cells) have negative charge. Energetic barriers are too high, indicating that physical attraction is not favored. Weak attraction between particles could occur at the secondary minimum (distances of 36 nm), for both AMZ and KAO API #5 there is no strong attraction between clays and cells. A physical mode of action driven by suffocation or mechanical tearing of the cell wall would probably 1) require higher attraction energy or 2) more particle density surrounding the cells.	**G.** Achieved. **H** Disproved.

#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.29 EM-ARM	**G.** Determine the composition of the dark particles observed around the cell. Determine the presence of Al, Cu or other transition metals inside the cell. This experiment looked at both physical and chemical modes of action. **H.** Al levels increase inside the cell.	Samples prepared for TEM (50 nm thickness) were observed in the JEOL ARM 200F aberration corrected TEM. The beam was operated at 80 KV. its resolution is at the atom level (0.8Å). This instrument was used to obtain Electron Energy Loss Spectroscopy (EELS) and Energy Dispersive Spectroscopy (EDS) on carbon-coated samples mounted in Au grids. Images were obtained in bright field, dark field and high angle dark field. The composition of the particles was analyzed with EDAX	EDS allowed recognition of clay nanoparticles (<100μm) able to interact with cell membranes and exo-polysaccharide substance (EPS, Fig 3.6). Si was detected inside the cell and Al and S were observed inside a cell feature (single-cell observation). EELS is not suitable to study my samples. The spectra showed C and O, but no other elements were above the detection limits of the instrument. The amount of material in 50 nm may have not been enough to be detected. The response of *B. subtilis* differed in that cells did not allowed alien particles through their membrane. Perhaps the thicker peptidoglycan layer provides a stronger barrier. Single–cell observations suggest that the cytotoxicity effect could be related to SiO_2, Al^{+3} and/or clay nanoparticles that compromise the membrane functions. However, it is not known if these single-cell results are significant.	**G.** Achieved for the minerals outside cells. **H.** Could not be confirmed by this method.
Experiments to test if the AMZ's Mode of Action is due to Chemical Interactions				
Experiments to study the chemical environment of the *E. coli*–clay suspension				
D.30 LB-Leach ICP-	**Q.** What is the composition of the antibacterial leachate? How does its chemistry compare to other antibacterial leachates? **H.** The leachate is not antibacterial because the concentration of elements is low.	The leachate was measured in 2 g clay concentrations: 50 mg/mL and 250 mg/mL. The samples were shaken for 24 h in a hand wrist shaker, the clays were centrifuged down at high speed and the supernatant was analyzed via ICP-MS.	The concentration of the elements in the leachate seems too low compared to the French and OMT clays. The elements are below MIC.	**Q.** Answered. **H.** Confirmed.

#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.31 FT-IR	**G.** Detect changes in clay after overnight incubation with LB and DI water. Test for dissolution occurring only in LB media **H.** Because the element concentration in LB was higher than the concentration in the exchange solution (with NH_4OAc), I hypothesized that the clay was dissolving with LB at 37 °C. if AMZ clay is dissolving with LB, and not with DIW, the FT-IR pattern will be different.	80 mg of clay were incubated overnight (37 °C) in 1mL of 5 g/L LB and DIW(in duplicates). The particles were settled by centrifugation and the clay was washed 3x with MilliQ water to remove media. The samples were diluted (~10 mg in suspension), and a drop was allowed to dry onto 2 mm thick ZnSe windows. FT-IR spectra was obtained in a Bruker IFS66V/S, using Mid-IR/ KBr beam splitter/ DTGS detector. The data was analyzed in OPUS, spectroscopy software.	The FT-IR patterns seem to be the same in both instances, indicating that the growth media does not modify the clay structure to detectable levels. *Note.* The data from the NH_4OAc exchange was not good. 1. the clay concentration was too low and 2. the molecule does not fully dissociate and it is not as effective as NaCl, or other salts of alkali metals.	**G.** Achieved. **H.** Disproved.
D.32 EQ3	**Q.** What is the element speciation in the AMZ leachate, and LB incubated with AMZ?	A text file was created with the concentrations (in M) of the ions (data from ICP-MS). Temp set to 37 °C, pH and Eh were set according to the values measured in the experiments after 24 h. The cations were balanced with Cl^-, the main ion in the leachate. The speciation was modeled in EQ3/6 software using the thermodynamic database: data0.sup.R2.	Element speciation was obtained. for the different dilution.	**Q.** Answered.
Experiments to test for the effect of pH				
D.33 pH	**G.** Determine the pH of the *E. coli*–AMZ system after overnight incubation (5 g/L LB). **H.** As with other antibacterial clays, the pH is acidic < 5.0. If the pH is neutral, or alkaline, the clay is not antibacterial.	Grow *E. coli* to 10^8 CFU/mL. Incubate cultures with clay (AMZ and controls). Measure the pH after 24 h. Minimum volume is 1 mL. Record the ORP or Eh, when available.	I started to monitor the pH after overnight incubation in the Fall of 2014. Eh was monitored later (in 2015), therefore I have less data for Eh. The average pH of the *E. coli* with clay suspension is 4.5 ± 0.1 n= 15. The mean of Eh = 400 mV ± 39. The pH of control *E. coli* was 8.3 ± 0.4 and the Eh = 300 mV ± 39.	**G.** Accomplished. The pH range was useful for experiments in which the LB was buffered. **H.** Confirmed.

#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.34 pH	**G.** Determine the impact of low pH on *E. coli's* viability. **H.** At pH 4.2, *E. coli* is not viable and the cells cannot grow.	Incubating *E. coli* (10^8 CFU/mL) in 5 g/L LB acidified (pH 4.2). For comparison, *E. coli* was incubated with 80 mg AMZ. *E. coli* incubated in neutral media was included for comparison.	The AMZ clay reduced the population of *E. coli* by half, as expected and had a pH of 4.5 after 24 h. The growth of *E. coli* was not inhibited in acidified LB, it was the same as the control, grown in neutral LB =10^9 CFU/mL. However, the pH of the acidified LB increased from 4.2 to 6.7. This experiment shows that either *E. coli* or the LB can increase the pH of an acidic solution to favor bacterial growth.	**Q.** Answered. **H.** Disproved. Gave me a good idea of pH limits that can be tolerated.
D.35 Acid media	**Q.** Can the antibacterial mode of action be explained by low pH? **H.** The effect of the AMZ clay on *E. coli's* viability will be greater than a buffer solution at the same pH of the AMZ.	Grow *E. coli* to 10^8 CFU/mL. Incubate cultures in 0.5 mL 0.1M Na-citrate-citric buffer (pH 4.5) mixed with 0.5 mL 10 g/L LB media. Incubate *E. coli* with AMZ (80 mg/mL), incubate a culture in acidified LB (not buffered) (pH 4.5)	Viability loss of 2 orders of magnitude of *E. coli* in buffered LB compared to 100% dead when *E. coli* pH after 24 h for buffer and AMZ was incubated with AMZ. 4.5. *E. coli* control had a pH 6 after overnight incubation (*E. coli* raises the pH).	**Q.** Answered. **H.** Confirmed.
D.36 Non-antibac.	**Q.** Could a non-antibacterial clay become antibacterial by acidifying it? **H.** Non-antibacterial clays could become antibacterial if they are acidified, and if a process of dissolution is catalyzed.	Incubating *E. coli* with non-antibacterial clays (SWy-1, KGa-1 and exchanged AMZ) + buffer (pH 4.5). Growing fresh culture to 10^8 CFU/mL then mixed 1mL culture with 50 mg clays KGa-1, SWy-1, exchanged AMZ (with KCl). Added 1mL citrate buffer (pH 4.5). Controls included the same non-antibacterial clays but without the buffer. Incubated overnight and plated. The pH and ORP were measured after 24 h. Experiments performed in duplicate.	All the samples incubated with the buffer showed a viability loss of 2 orders of magnitude, independent of the clay. The non-antibacterial clays without a buffer showed similar growth to the *E. coli* without a buffer. The design of the experiment did not test what I intended. The viability loss is due to the pH, but cannot be linked to clays.	**Q.** Results are not conclusive. Problematic design.
D.37 EDTA	**G**. Determine if adding a metal chelator (EDTA/ oxalate buffer) changes the antibacterial properties of clay. **H**. if the antibacterial mode of action is due to species in solution, adding a metal chelator would stop the antibacterial property.	50 mg of AMZ clay were autoclaved and mixed with 1 mL of *E. coli* (10^8 CFU/mL) in log phase and 1 mL of 0.5 M EDTA. Because EDTA/ox buffer raised the pH of the solution (pH 7.6), the experiment was conducted in buffered LB media (1 mL of 0.1 M citric-citrate buffer was added, pH 4.2). The following samples were tested: AMZ+ *E. coli* + EDTA/ox; KGa-1+ *E. coli* + EDTA/ox; *E. coli* + EDTA/ox+ buffer (controls).	In all samples (including controls) the viability of *E. coli* decreased by half. I interpreted that the low pH (4.2) decreased viability of cells. Adding a buffer was necessary, because at pH > 5.0 the AMZ clay is not antibacterial. However, the pH alone can reduce the population, and in this case, the pH 4.2 may have been too low. A pH of 4.2 may be the MIC of log H^+ concentration.	**G.** Not achieved. **H.** Could not be tested.

Experiments to test for metal toxicity				
#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.38 MIC/ MBC Al	**Q**. What is the MIC/MBC of Al at pH 4.5? How does it compare to the levels found in the exchange–solution, the LB incubated with AMZ? **H.** The MIC of Al decreases at lower pH. The Al found in the LB incubated with AMZ may be above the MIC.	Media: 5g/L LB. Aluminum stock 50 mM $AlCl_3$ solution. *E. coli* was grown to exponential log phase in 5 g/L LB. Aliquots of 1 mL fresh culture (10^8 CFU/mL) were transferred into Eppendorf tubes and cells were harvested. After pelleting the cells, the neutral LB was discarded and cells were re-suspended in the acidified LB, followed by addition of X µL of the metal stock needed to reach final concentrations of 0.5, 1.0 and 2.0 mM Al. Viability test. A control population of *E. coli* incubated in the acidified LB was included. Experimental triplicates. The pH was measured in the *E. coli* cultures after 24 h.	The Al in LB incubated with AMZ is twice the MIC. The MBC concentrations are not exceeded in the experiments.	**Q.** Answered **H.** Confirmed.
D.39 MIC/ MBC	**G.** Determine the MIC/MBC of Cu at pH 4.5. **H**. The Cu found in the LB incubated with AMZ may is above the MIC.	Same procedure as outlined for Al. Cu stock = 10mM, prepared with $CuCl_2$. Concentrations tested: 0.05, 0.5 and 1.0 mM Cu.	The Cu in LB incubated with AMZ is below MIC by half.	**G.** Achieved **H**. Not Confirmed
D.40 MIC/ MBC	**G.** Test for the effect of a consortia of metals derived from the AMZ. **H**. A mixture of metals, all of them below MIC, will effectively produce 100 % viability loss in *E. coli.*	Fresh culture of *E. coli* grown in neutral 5 g/L LB and resuspended in acidified LB. Metal cocktail stock diluted in liquid cultures (1:100, 1:500, 1:1000). Viability test performed.	The *E. coli* did not survive incubation with the metal cocktail at the concentrations found at the LB with AMZ (Figure 4.6), neither by this concentration diluted 5 times, and it was inhibited by a 10^{th} of the concentration of the metal cocktail (Figure 4.6).	**G**. Achieved **H.** Confirmed

#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.41 EQ3	**G**. Model the metal speciation in the metal cocktail (as a proxy for the chemical environment created by the AMZ clay). **H.** Al^{3+} will dominate the Al speciation.	A text file was created with the concentrations (in M) of the ions. Temp set to 37 C, pH and Eh were set according to the values measured in the experiments after 24 H. The speciation was modeled in EQ3/6 software using the thermodynamic database: data0.sup.R2.	The species were modeled at the concentrations found in the LB incubated with AMZ, and also at the MIC and MBC concentrations of the metal.	**G.** Achieved **H.** Confirmed
D.42 NanoSIMS	**G.** Investigate the distribution of elements in *E. coli*–treated–with–AMZ compared to a control *E. coli*. **H.** Al is inside the cells as well as in the membrane. Cu and Fe are elevated in *E. coli* reacted with AMZ.	Ion images of carbon, aluminum, iron, and copper were performed on *E. coli*, with and without incubation with AMZ clay via NanoSIMS	Results showed greater accumulation of Al and Fe in AMZ–treated cells. The accumulation for Al is greater at the membrane, while the accumulation for Fe was greater inside the cells.	**G.** Achieved. **H.** Confirmed
Experiments to Test for Reactive Oxygen Species				
D.43 H_2O_2	**G.** Test if the AMZ clay produces considerable amounts of H_2O_2 compared to OMT antibacterial clay. **H.** If the H_2O_2 production is at least 100 µM, then the mode of action may be related to ROS production	Weigh 80 mg of clay (AMZ, KGa-1) in 9 Eppendorf tubes each sample. Add 1 mL 0.85% NaCl, (saline) include a tube (x3) with a blank of saline. Incubate at 37°C. After 2 h centrifuge down the clays and pipette out 700 µL of supernatant into another Eppendorf tube. Add 200 µL buffer, 50 µL LCV, 2 µL 0.5 EDTA, and 50 µL HRP. Incubate for 20 min in the dark, at room temperature. Measure the absorbance at 590 nm. Do the same after 6 h and 20 h, with the other micro centrifuge tubes incubated (Cohn et al., 2005).	H_2O_2 production by AMZ clay was 0.3 ±0.1 µM after 2 h, and it increased to 0.38 ± 0.01 after 20 h. The standard kaolinite did not produce H_2O_2. AMZ produces H_2O_2, but concentrations above 1 mM H_2O_2 are required to affect E. coli. The OMT clay produces 100 µM H_2O_2 and this concentration remains relatively constant after 24 h	**G.** Achieved **H**. Not confirmed.

Experiments to Determine if Nutrient Starvation (i.e. P and Mg) reduce viability in E. coli				
#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.44 Adding P	**Q.** Does supplementing the media with nutrients increase the survival of *E. coli?* **H.** if the antibacterial mode of action is related to nutrient starvation, the controls supplemented with P, or P + Mg will survive significantly more than cultures that do not receive the nutrients.	*E. coli* was incubated with 70 mg/mL AMZ clay for 2 h. Then, P was added to cultures, as NaH_2PO_4. Because a decreased in Mg was detected in *E. coli* cells (Table 3.7); Mg was also supplemented to the media, along with P.	The *E. coli* population declined by three orders of magnitude regardless of the addition of PO_4^{3-} (Figure 4.5). Therefore, depletion of phosphate is not a factor that governs the decline in bacterial survival.	**Q**. Answered. **H.** Disproved.
D.45 AP	**Q**. Is *E. coli* activating responses to P starvation? **H**. If the alkaline phosphatase enzyme is high in *E. coli* treated with AMZ, the *E. coli* does not have enough P available, and that could halt its growth.	Expose bacteria to clay and conduct AP assay to detect the activity of the enzyme (for a more comprehensive and detailed explanation, please refer to the Appendix E–Other experiments).	The AP test in the presence of AMZ gives results that are difficult to interpret. The background concentration of AP was in cells treated with AMZ is lower than the background (the enzyme produced by *E. coli* that is not under P starvation). While it is possible that there is no P starvation in *E. coli*–reacted–with–AMZ, the levels below background may indicate adsorption of the enzyme, or inactivation due to metals. In addition to that, the alkaline phosphatase enzyme is not stable at low pH.	**Q.** Not answered **H.** Could not be tested.

Experiments to determine the targets of AMZ (i.e. membrane or DNA)				
#	Goal (G), question (Q), or hypothesis (H)	Method	Results overview	
D.46 X-gal	**Q.** Is the AMZ damaging the envelope? **H.** If the AMZ clay damages the envelope, the *E. coli* susceptible of membrane damage will not be able to grow in the neighborhood of the well.	An *E. coli* mutant, sensitive to envelope damage was spread onto 'sloppy' agar plates (greater water content so things can diffuse). Wells were created to place the hydrated clay, to test for its antibacterial effect. The plates were incubated overnight.	No zone or ring of inhibition was observed. In previous trials, it was noted that the 'zone of inhibition' method was appropriate. This remains true, even though the water content increased.	**Q.** Not answered by this method. **H.** Could not be tested, method is not appropriate.
D.47 Vancomicin	**Q.** Is the membrane integrity of *E. coli* compromised? **H.** If the membrane permeability increases, the viability loss will be greater in *E. coli* incubated with vancomycin and a sub-lethal dose of AMZ, than the viability loss caused by the same dose of AMZ	*E. coli* was grown in 5 g/L LB. Fresh cultures in exponential log phase were incubated with 50 mg/mL AMZ and 40 µg/L vancomycin, and compared to *E. coli* incubated only with 50 mg/mL AMZ, and *E. coli* with 40 µg/L vancomycin. *E. coli* without treatments was grown as a control. This experiment was performed several times. For a comprehensive description of the experiment and results, please refer to the Appendix B–Other experiments	Results showed that the vancomycin plus the AMZ decreased the viability of *E. coli* significantly more than *E. coli* treated with AMZ, or *E. coli* treated with vancomycin alone ($p = 0.05$, ANOVA)	**Q.** not answered. **H.** confirmed
D.48 Nitrocefin	**Q.** Is the Is the membrane integrity of *E. coli* compromised? **H.** If the outer membrane of *E. coli* is damaged, then periplasmic enzymes will be released and detected by a chromophore molecule.	The *E. coli* strain RAM 2523, resistant to β-lactam antibiotics such as nitrocefin) was cultured and exposed to AMZ. Different modifications of the method were tried to measure the enzymes released from the periplasmic space. For a comprehensive description of the experiments and results, please refer to the Appendix B–Other experiments	Results showed that the *E. coli* incubated with AMZ released less enzymes than the control *E. coli*	**Q.** not answered **H.** could not be tested using this method.

APPENDIX E

OTHER EXPERIMENTS

Experiments to Determine if the AMZ Clay Damages the Membrane

Membrane Permeability Using Vancomycin and 50 mg AMZ

Question. What is the appropriate concentration of vancomycin needed to see an effect on *E. coli* if the concentration of clay is slightly below the minimum inhibitory concentration (MIC.) The clay concentration should be sub-inhibitory, enough to initiate membrane damage (if present) so vancomycin can pass through outer membrane and attack the petidoglycan layer in the cell, increasing cell death.

Method. Two *E. coli* cultures (12 mL each) were grown to mid-exponential phase in 5 g/L LB and to a concentration of 10^8 CFU/ml. The fresh culture was distributed in four glass tubes, in volumes of 2.2 mL each (See Figure E1). The first 2.2 mL were further distributed into two Eppendorf tubes (1 mL each), and labeled control *E. coli*. For the second tube, the 2.2 mL were further distributed into 2 tubes containing 50 mg AMZ each. The 2.2 mL of the third and fourth tubes were mixed with vancomycin (final concentrations of 40 µg/mL and 80 µg/mL respectively). After mixing the vancomycin into the cultures, 1 mL of the mixture was pipetted into tubes containing 50 mg clay (in duplicate). After overnight incubation, the samples were serially diluted and plated. Experiments were performed in two different cultures and each culture had duplicates (2 experimental duplicates and 2 technical duplicates).

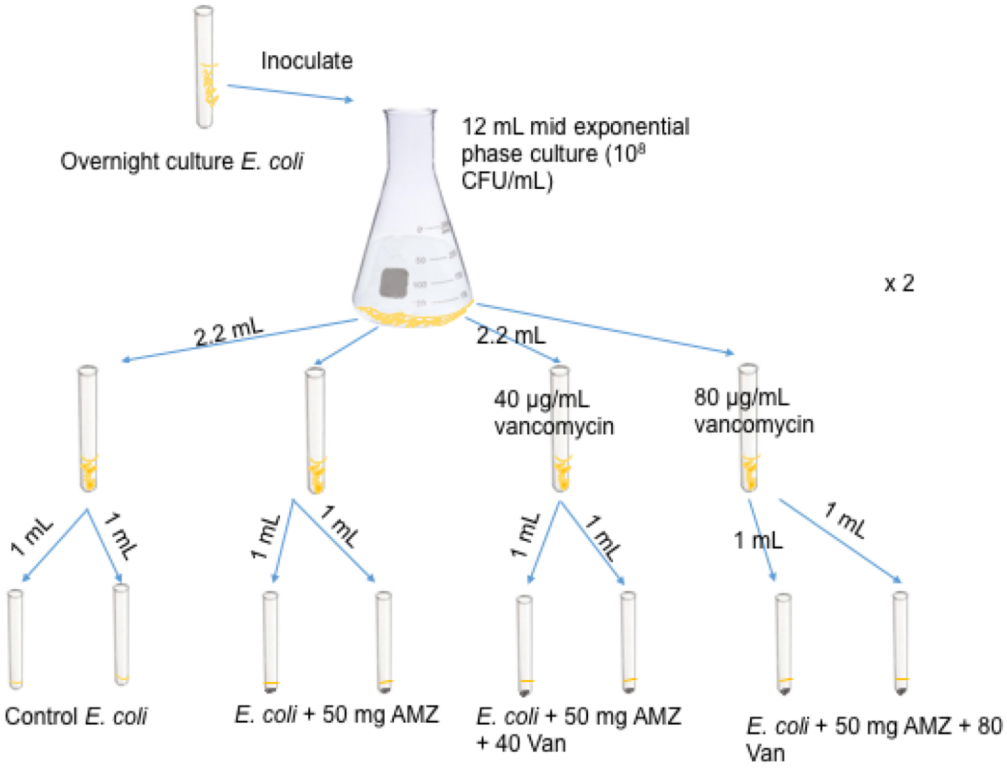

Figure E.1. Representation of the experimental design for the vancomycin tests. The vancomycin was mixed into 2.2 mL culture to make a homogeneous dilution in the technical duplicates.

Results. The AMZ clay (50 mg/mL) decreased the viability by one order of magnitude of *E. coli*. This contrasts with the viability loss of *E. coli* when it was incubated with 50 mg AMZ and two different doses of vancomycin, which produced a reduction of four orders of magnitude (Figure E2).

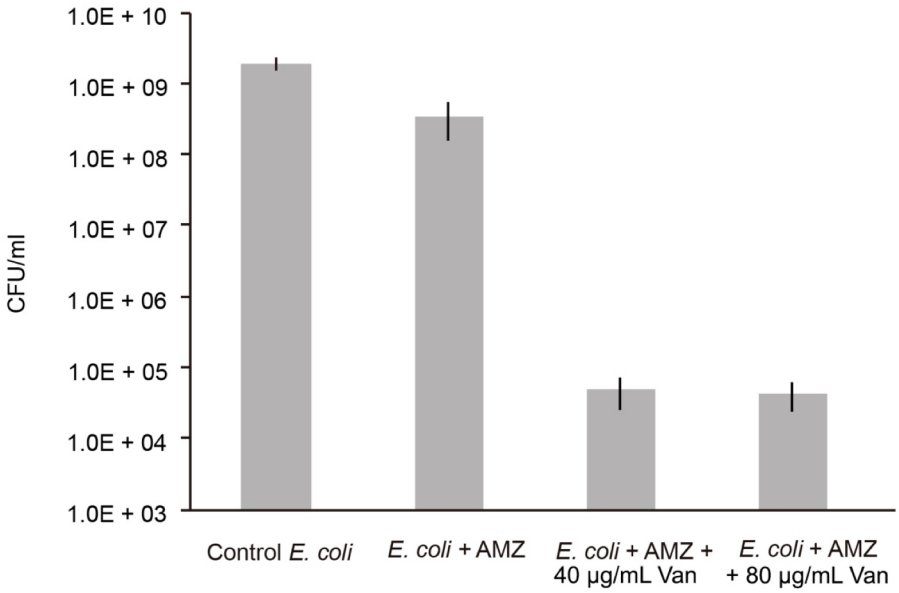

Figure E.2 Results of exposing *E. coli* to 50 mg/mL AMZ, and 50 mg/mL AMZ with two doses of vancomycin (40 µg/mL and 80 µg/mL).

Conclusion. This result suggests that the membrane of *E. coli* is being permeabilized by the AMZ clay. However, the effect of vancomycin alone was not assessed. The test will be repeated including a control for the effect (if any) that vancomycin has on *E. coli* cells.

Membrane Permeability Test Using 40 µg/mL Vancomycin and 50 mg AMZ (including control for vancomycin effect)

Goal. In this experiment the clay concentration the same dose of AMZ was used (50 mg), the vancomycin concentration used was 40 µg/L vancomycin. Results were compared to *E. coli* without treatment, *E. coli* with 40 µg/mL vancomycin and *E. coli* with AMZ alone.

Method. Same procedure outlined in Figure E1 but using only one concentration of vancomycin, and including a control for the vancomycin compound.

Results. Results showed that the viability of *E. coli* incubated with a sub-lethal dose of AMZ (50 mg/ml) plus vancomycin was two orders of magnitude less than the viability loss produced by 50 mg/ml of AMZ alone, and three orders of magnitude lass than the control *E. coli* (Figure E3 and Figure 4.10). The dose of vancomycin (40 μg/mL) plus AMZ had a significant effect on *E. coli* at the $p < 0.05$ level according to an ANOVA analysis of variance. The vancomycin alone has en effect on *E. coli* cells that is comparable to a sub-inhibitory dose of AMZ clay.

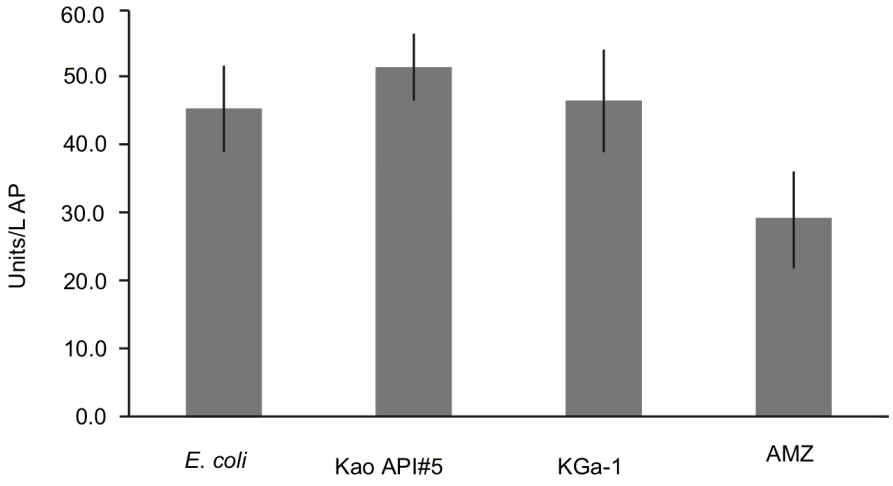

Figure E.3 Results of viability count performed to confirm the antibacterial activity of the clay.

Conclusions. Results confirmed that the membrane is permeabilized using 50 mg of clay. The control for the vancomycin was included.

Membrane Permeability Test to Detect Enzyme Leaking Using Nitrocefin

Description. A series of colorimetric assays aimed at detecting β-lactamase enzymes released from the periplasmic space of *E. coli* (O'Callaghan et al., 1972).

Rationale. Enzymes produced in the periplasmic space will leak through the outer membrane if it fails to contain them, due to increased permeability of damage due to the AMZ clay. The *E. coli* strain RAM 2523 is resistant to β-lactam antibiotics (e.g., ampicillin and nitrocefin), it produces a β-lactam enzyme that cleaves the antibiotic in its periplasmic space (Richmond, 1973). If the outer membrane is permeabilized or damaged, the enzyme levels should be significantly higher in the media of *E. coli* treated with AMZ compared to a control *E. coli*. Nitrocefin reports the levels of the β-lactam enzyme in the supernatant. Cleavage of nitrocefin by the β-lactamase enzyme shifts its color from yellow to red. The presence of the hydrolyzed product can be measured in a spectrophotometer between 380 to 500 nm (O'Callaghan et al., 1972).

The hypothesis in this experiment is that if the AMZ clay affects the outter membrane, more β-lactamase will be released from the *E. coli* (strain RAM 2423) reacted with AMZ compared to two controls: *E. coli* reacted with a reference kaolinite and *E. coli* alone.

Method (variation 1). The strain RAM 2523 was kindly provided by Dr. Rajeev Misra. The strain was cultured in 5 g/L LB containing ampicillin (1 μg/mL) in order to select only the ampicillin-resistant strains. Fresh cultures were grown to 10^8 CFU/mL exponential growth phase. One mL of culture was exposed to a sub-lethal concentration of clay (50 mg AMZ/mL; the cells need to be metabolically active in order to produce β-

lactam) and incubated at 37 °C for 4 h. Controls included *E. coli* RAM 2523 reacted with KGa-,1 and *E. coli* RAM 2523 alone. After incubation, 500 µL of the suspension was transferred into Eppendorf tubes and centrifuged to pellet cells and minerals, (13,000 g for 2 min). Then, 200 µL of the supernatant were transferred into micro-triter well plates, in duplicates, to test for the presence of the β-lactam enzyme. 20 µL of nitrocefin (1 µL/mL) were pipetted into each well. The absorbance at 460 nm was read in kinetic mode for 30 min in a micro plate reader. In addition, a viable test count was performed in order to corroborate the antibacterial action of the clay. A blank with just LB was also included.

Results. The results are shown in Figure B4. The levels of β-lactam in the samples of *E. coli* reacted with clays were significantly lower than the levels detected in the control *E. coli* (i.e. the background level). The levels detected in the *E. coli* reacted with AMZ were below the blank (LB), no color shift was noted, suggesting that there was no enzyme in the supernatant. The color of the LB produced a number in the spec. The supernatant of the *E. coli* reacted with KGa-1 turned orange, and it was more pale than the supernatant of the control *E. coli.* Certain levels of enzyme in the supernatant are expected. The control *E. coli* without treatment defines the baseline. In this experiment, treatment with both clays resulted in levels lower than the control, which may be an artifact of the minerals. Therefore, the method was modified.

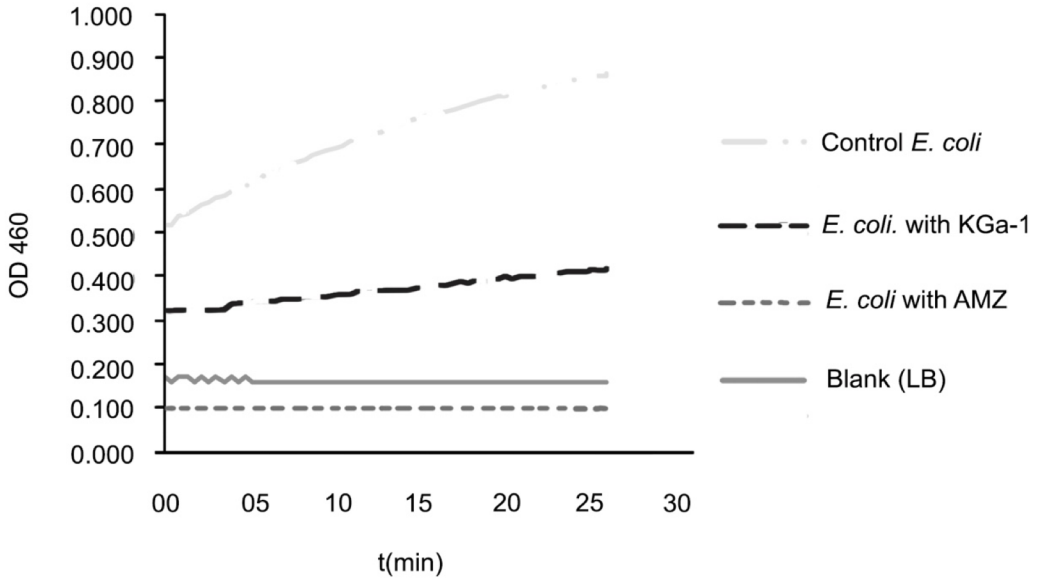

Figure E.4. Results of the nitrocefin assay performed in a supernatant from *E. coli* incubated with 50 mg of clay and *E. coli* alone.

Viability count results. The viability count showed that the AMZ clay inhibited the *E. coli* population by two orders of magnitude compared to the *E. coli* reacted with KGa-1 and to the control *E. coli.* Although the AMZ inhibited growth, the results of the nitrocefin test did not detect β-lactam enzyme. Since the enzyme was detected in the control *E. coli* it is concluded that the minerals are interfering with the assay. One mechanism is by delivering metals that inactivate the β-lactam enzyme. Alternatively, the enzyme was not in the supernatant because it was adsorbed to the surface of clay minerals. However, the cephalosporin is a non-polar compound, and kaolinite does not

have important surface charge, therefore the metals released form the clay may be having an effect.

Method (variation 2.) The idea was to do a preliminary test metals released by the clay were chelated with 0.5 M EDTA. The RAM 2523 was cultured as outlined above (in 5 g/L LB media with ampicillin). Then 50 mg of clay (AMZ and KGa-1) were mixed with 1 mL of culture and incubated for 2 h. Then, 500 µL of the suspension were withdrawn and centrifuged at high speed, 200 µL of the supernatant were transferred into an Eppendorf tube and allowed to react for 30 min with 200 µL of 0.5 M EDTA. After 30 min, the sample was transferred into a microplate and 20 µL of nitrocefin were added to each well. The shift in color was monitored at λ= 460 nm for 30 min.

Results. Addition of EDTA increased the detection of β-lactamase enzyme produced by the *E. coli* reacted with AMZ, to the same level as the control *E. coli*. Similarly, the detection of enzyme in *E. coli* reacted with KGa-1 increased compared to method var. 1, but the levels did not reach the baseline (Figure E.5).

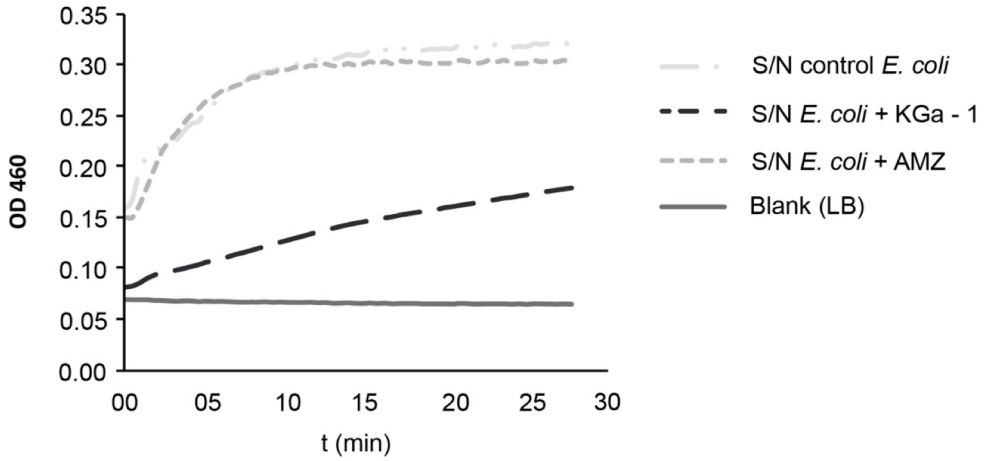

Figure E.5. Results of adding EDTA to supernatant of *E. coli* reacted with clays and *E. coli* alone

Method (variation 3.) Repeat the procedure of the variation number 2, but instead of adding EDTA to a supernatant, add EDTA to the suspension of *E. coli* and clay. Let react for 30 min. Then pellet down cells and minerals, and repeat procedure to monitor reaction with nitrocefin in microplate wells in supernatant.

Results. Results differed from the previous variation. Adding the EDTA to the clay/cell suspension increased the enzyme detection in the *E. coli* reacted with KGa-1 but not in the *E. coli* reacted with AMZ, the last one was lower than the baseline. It is possible that a more concentrated EDTA solution is needed to chelate the metals in the AMZ. Regardless, the levels of enzyme are not above the *E. coli* control, which could mean that membrane is not increasing its permeability, or that this is an artifact produced by an interaction between the clay and the molecules (Figure E6).

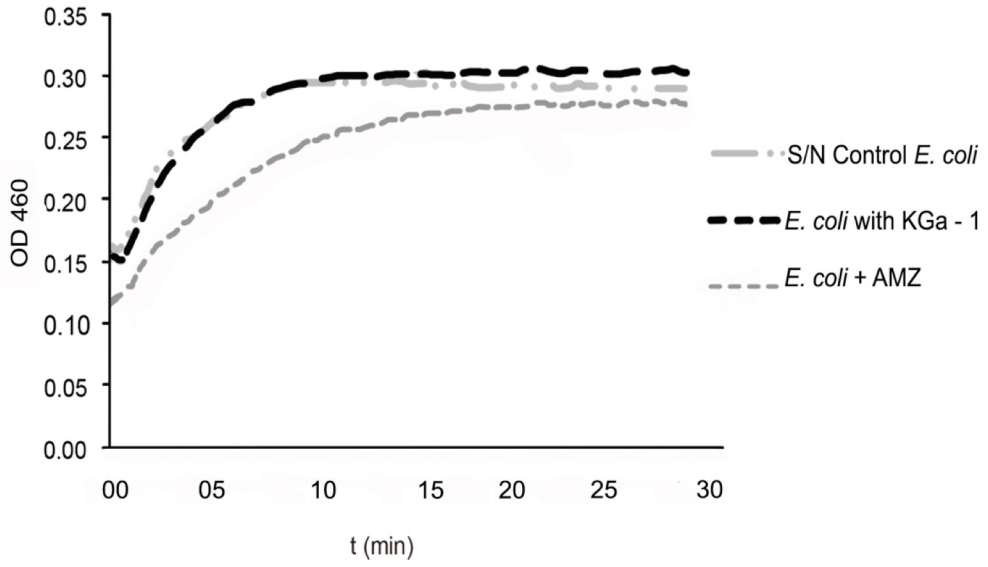

Figure E.6 Results of adding EDTA to the clay- *E. coli* suspension previous to performing the nitrocefin assay.

Method (variation 4.) In this variation 50 mg of clay will be mixed with a supernatant containing the β-lactam enzyme. The idea is to test if the clay is adsorbing the enzyme. First, 5 mL of L *E. coli (*RAM 2523) were grown to 10^8 CFU/mL in 5 g/L LB containing ampicillin. The cells were pelleted and the supernatant was decanted in a different tube. The supernatant should contain the enzyme naturally produced by this strain in the presence of β-lactam antibiotics. Then 1 mL of the supernatant was mixed with 50 mg clay (AMZ and KGa-1) in Eppendorf tubes, a control of the supernatant alone was included. The tubes were incubated at 37 °C for 2 h, followed by centrifugation to sediment the minerals. 200 μL of the supernatant were plated in micro-plates, each well received 20 μL nitrocefin and the absorbance at 460 nm was measured as before.

Results. No enzyme was detected in the supernatant mixed with clays. The control supernatant showed a continuous increase in the reaction (Figure B7).

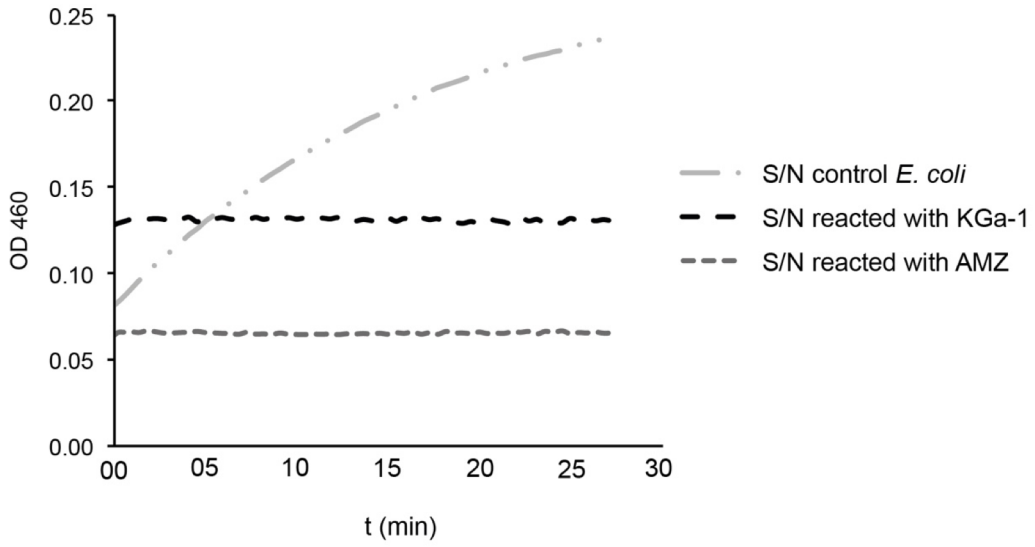

Figure E.7. Results of mixing the β-lactam enzyme in supernatant with AMZ clay No enzyme is detected after 2 h incubation.

It is interpreted that the clays retained the enzyme, and no enzyme was left to react with nitrocefin in the supernatant.

Conclusion. Activity of the β-lactam enzyme was detected only by adding EDTA, and different results were obtained when the EDTA was added to the supernatant, or to the clay mineral suspension. It is uncertain to what extent was the EDTA able to restore the enzymatic reaction, that is, how much of the enzyme remained unable to react

with nitrocefin after the EDTA was added. The results are highly interpretative. Therefore, this test is not considered suitable for clay-bacteria suspensions.

Experiment to Test If *E. coli* Faces Phosphate Starvation due to the AMZ Clay

Preamble. Alkaline phosphatase (AP) is an enzyme produced by bacteria, (e.g., *E. coli*; Garen & Levinthal, 1960) under P limitation. The enzyme increases P availability by cleaving phosphate groups from compounds, a property that is exploited in this test. The AP cleaves ρ-Nitrophenyl phosphate (ρNPP) into p-nitrophenol (ρNP), releasing a phosphate group that can be used by the cell. (Figure E8)

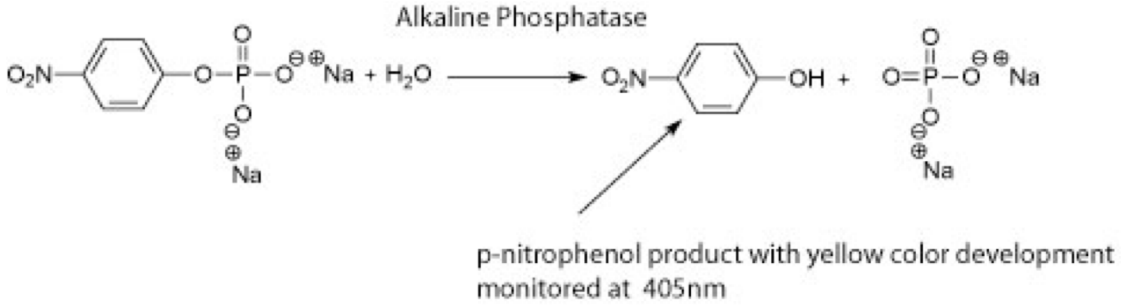

Figure E.8. Reaction between ρ-Nitrophenyl (pNPP) and alkaline phosphatase (AP) produces p-nitrophenol (pNP) and frees (Image modified from Sigma Aldrich, http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/alkaline-phosphatase.html)

The amount of ρNP produced can be measured with an UV spectrophotometer at $\lambda = 405$ nm, the product has a yellow color; the ρNP can be related to the alkaline phosphatase produced by a standard curve, as explained below.

Method. The method was modified from Tabatai & Bremner (1969), a method to detect phosphatase activity in soils. Poret-Peterson, Steger and Lee, from the astrobiology group at ASU, included an alkaline phosphatase control or AP standards (Poret-Peterson, *pers. comm*). First, a curve of ρ-Nitrophenol (pNP) concentration vs. optical density at 405 nm (OD_{405}) was generated by reacting a series of dilutions of the AP enzyme with ρNPP. Briefly, an AP stock (Sigma cat No. p5521, SLB6-3802V; diluted in Tris HCl/ $MgCl_2$) was prepared by diluting the AP in 50 mM Tris-HCl and MilliQ water to a concentration of 385 ng/L. From this, a series of 10 fold dilutions were prepared, called here the AP standards. The general assay was performed by mixing 890 µL Tris HCl/ $MgCl_2$, 100 µL of ρNPP (in 10x TRIS HCl/ $MgCl_2$) and 10 µL of sample (AP standard, in this case). The samples were incubated at 37 °C for 30 min. 50 µL of NaOH were added to stop the reaction after 30 min and the absorbance at 405 nm was read. A preliminary, quick test to see if clay minerals interfered with the assay was performed by incubating for 30 min, 40 mg of AMZ clay with 500 µL of the AP standards, and 10 µL ρNPP. The suspension turned yellow, that is, the reaction between the AP and ρNPP proceeded in the presence of clay under this conditions. Thus it was interpreted that the AMZ would not interfere with the reaction.

The protocol had to be adapted to test *E. coli* instead of the pure enzyme. First, the ideal biomass of *E. coli* was determined as follows: *E. coli* was cultured in 5 g/L LB to different densities (10^6 –10^9 CFU/mL.) The cells were lysed by suspending them in 300-500 µL of B-PER (a reagent for bacterial protein extraction; the *E. coli* cells were lysed in B-PER because the TRIS HCl solution did not produce good lysing), shaken for 20 min and centrifuged to pellet the debris. Then the protein concentration in the

supernatant was determined with the BCA protein assay (Thermo scientific protocol NanoDrop 2000). The AP assay was performed on the cell lysate. It was determined that a culture of 10^8 CFU/mL would yield enough protein for the assay. Therefore, a new calibration curve (Figure E.9) was done using the AP standard diluted in B-PER.

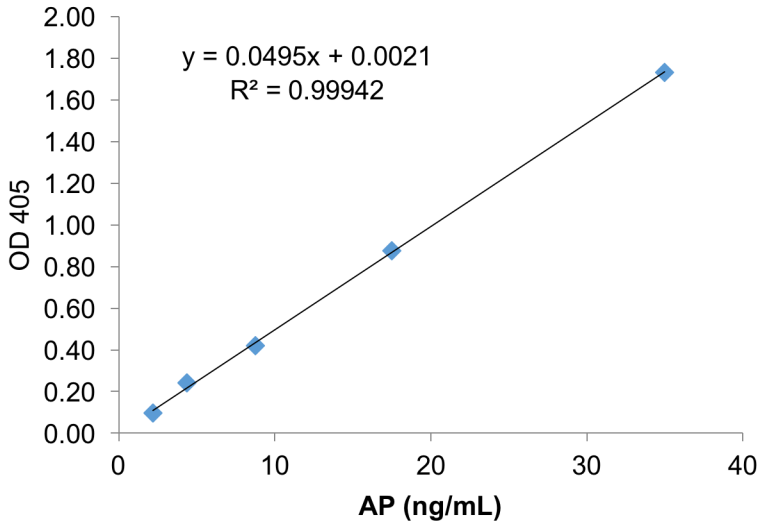

Figure E.9 Calibration curve of alkaline phosphatase (AP) standards in TRIS

Next, the AMZ clay was assayed with the AP standards (dilution series) to see if the AP enzyme activity was detected. Briefly, 100 µL of AP standards were incubated with either 10 mg or 8 mg of clay in Eppendorf tubes. Blanks for only clay and only AP were included. After 30 min of incubation, the clays and enzyme were centrifuged down and the assay was performed on the supernatant (S/N; 50 µL S/N + 850 µL Tris HCl + 100 µL ρNPP, incubated for 60 min). There was no color change in the samples. Results could mean that the AP standards had been adsorbed by the clay, and therefore were not

present in the supernatant, or, that clay had inactivated the enzyme and it was no longer able to react with the ρNPP (Table E.1).

Since the test could not be performed in the supernatant, the assay was performed by adding the reagent directly to the tube containing the AMZ clay and the AP enzyme

Table E.1

Alkaline Phosphatase activity using two AMZ clay concentrations (ρNPP Assay)

Clay Concentration:100mg/mL		Clay concentration: 80mg/mL	
AP (ng/mL)	OD 405	AP (ng/mL)	OD 405
70	0.085	70	0.158
35	-0.023	35	0.051
17.5	0.002	17.5	-0.002
8..8	-0.011	8..8	-0.024
4.4	-0.027	4.4	-0.046
2.2	-0.047	2.2	-0.063
1.1	-0.018	1.1	-0.05
AMZ (no AP)	-0.022	AMZ (no AP)	-0.049

(100 mg clay/mL liquid). The detection of the enzyme increased when done in this way (Table E2).

Table E.2

Alkaline Phosphatase Activity by modifyng the protocol

Clay Concentration:100mg/mL		
AP (ng/mL)	OD 405	SD
70	***	
35	1.220	0.36
17.5	0.510	0.30
8.8	0.371	0.04
4.4	0.118	0.06
2.2	0.037	0.04
1.1	0.007	0.03
0	-0.028	

Note. *** Out of range

This assay performed with the AP standards shows that if there is enough AP in the suspension, the assay would detect the enzyme. However, the amount of enzyme detected is less when the clay is present than without the clay (Figure E10), even though the same standard was used.

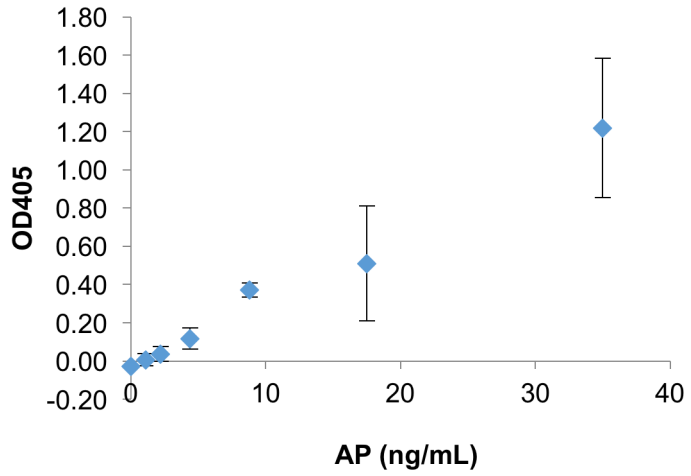

Figure E.10 Callibration curve of Alkaline Phosphatase using B-PER extraction reagent.

The next experiment was performed with cells, instead of the free enzyme. To increase the enzyme detectability, the cells were lysed in the clay-bacteria suspension, and the assay was performed in the tube, along with the clay, based on the previous experiment. The concentration of the clay was reduced to 80 mg/mL and standard kaolinites (KGa-1 and KAO API#5 were included as controls). Briefly, *E. coli* was cultured in 5 g/L LB to a concentration of 10^8 CFU/mL. Then 1 mL of culture (exponential log phase) was mixed with clay (AMZ and KGa-1) and incubated overnight.

The next day, the cells and minerals were pelleted, resuspended in 500 µL B-PER to lyse the *E. coli* followed by high speed centrifugation to pellet the cells and minerals. The assay was performed with 100 and 350 µl of sample separately and the samples were incubated for 3 h, since no change in color was observed after 30, 60, and 90 min.

The OD 405 was converted into µmol ρNP using the calibration curve. The activity of alkaline phosphatase (AP) in the samples is calculated as:

$$AP\ \left(\frac{Units}{mL}\right) = \left(\frac{A}{V}\right)/T$$

A = amount of ρNP generated in samples calculated from standard curve (µmol)

V = volume of sample added in the assay well (mL).

T = reaction time (minutes).

Results showed that the AP activity is less than the control *E. coli,* which is the baseline of AP production, in cells that are not P-starved. The enzyme activity in control clays is equivalent to that of the control.

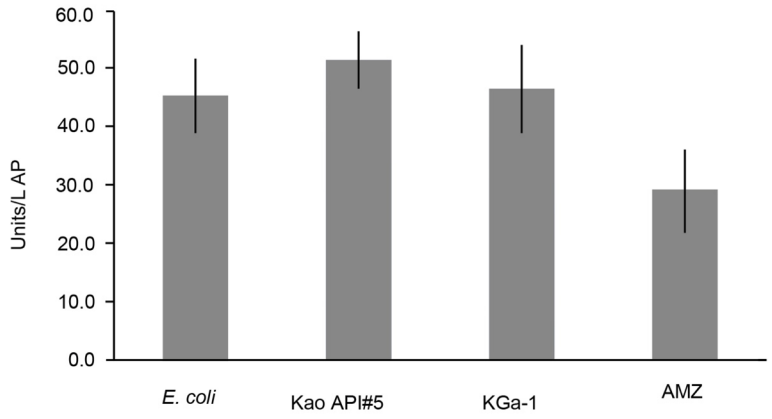

Figure E.11 Viability test performed to confirm the antibacterial effectiveness of the AMZ clay during the experiment

The AP test in the presence of AMZ gives results that are difficult to interpret. At least the background concentration was expected. While it is possible that there is no AP production, i.e., there is no P starvation in *E. coli*–reacted–with–AMZ, the levels below background may indicate adsorption of the enzyme, or inactivation due to metals.

Conclusion. This test is not appropriate to test for P starvation.

APPENDIX F

PLACE-BASED SAMPLE UNITS

UNIT 1: EARTH SYSTEM'S IN NORTHERN OF SOUTH AMERICA

Subject Area: Natural Sciences

Length of unit: Four classes

Brief description of unit

The unit is designed for 4th grade students. The unit covers geological changes in northern South America that configured the Amazon Basin.

<table>
<tr><th colspan="2">STAGE 1 - DESIRED RESULTS</th></tr>
<tr><td rowspan="4">Established Goals Standards.

This unit addresses Colombian competencies: Establish relationships between plate tectonics, topography and the forces that generate them.

and the NGGS- 4-ESS2-2

Specific goals.

• Students will understand the concepts of erosion, weathering, and deposition and how those processes have acted to form the Amazon rain forest.
• Students will work with an oral tradition story to think about the</td><td>Enduring Understanding</td></tr>
<tr><td>Earth is continuously changing (Earth science big idea #4; ESLI, 2010). The natural world can be studied from multiple perspectives.</td></tr>
<tr><td>Essential Questions</td></tr>
<tr><td>• How do erosion, weathering, and deposition shape a landscape?
• Why are some regions like the Andes high in elevation?
• How are the Andes connected to the Amazon River?
• How did Amazonia form according to Western and to native science?
• How did the Amazon form according to the story of the <u>Moniya Aména</u> tree?</td></tr>
<tr><td>Essential Ideas</td></tr>
<tr><td>4-ESS2.A: Earth Materials and Systems. Rainfall helps to shape the land and affects the types of living things found in a region. Water, ice, wind, living organisms, and gravity break rocks, soils, and sediments into smaller particles and move them around. (4-ESS2-1; NGSS, 2013, p.40)

ESS2. B. The locations of mountain ranges, ocean floor structures, and rivers, occur in patterns. Major mountain chains form inside continents or near their edges (4-ESS2-2). Most rivers organize themselves in connected networks that can resemble a tree from space.

Uitoto knowledge. Traditional explanations in narratives capture the functioning of Earth and ecosystems. Sometimes, even the patterns of natural features recognized on Earth can be transmitted in traditional stories.</td></tr>
</table>

origin of the Amazon.	

STAGE 2 – EVIDENCE

Assessment Evidence

Performance expectations

4-ESS2-2. Analyze and interpret data from maps to describe patterns of Earth's features. [Clarification Statement: Maps can include google Earth or topographic maps of Northern South America that show the Pacific and Atlantic Oceans, the Andes, and the Amazon basin.]

Other assessments

"I Learned" statements: Before class ends, with perhaps three minutes remaining, the teacher asks students to write "I learned..." on a sheet of paper and then complete the sentence (Bond & Evans, 2011).

Ask students to write three statement beginning with: "I would like to learn more about…"

STAGE 3 – LEARNING PLAN

Learning Activities

1. To introduce the unit, follow the suggested activity # 1 (adapted from Reynolds et al., 2010)
2. Tell the students the story of the *Moniya Aména*, emphasizing the key events that will help them link it to the origin of the Amazon (Londoño et al., 2016).
3. Perform the 'Weathering, erosion and deposition activity' (from Heaton, 2014)
4. To teach plate tectonics and large-scale system interactions perform activity 2 (Watts & Hayde, 2014).
5. Tell the students the Western science model of the origin of the Amazon (Hoorn, 2006)
6. Perform an assessment for unit completion: Integrating knowledge tets.

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Identifying Features from Space

(Suggested Activity # 1)

Begin by showing the students the Google Earth map of South America (Figure 1). Remember to indicate the cardinal directions (N, S, E, and W). Ask your students to observe the image in detail.

Figure C.1 Google Earth image of Northern South America

Ask students to:

- Describe the image in their notebook, or redraw it noting all the aspects that caught their attention.
- Explain what the different colors mean in the image.

- Identify some of the features (e.g., Andes Mountains, Pacific and Atlantic oceans, Amazon Rainforest, Amazon River)
- Observe the locations of the Pacific Ocean, the subduction zone, the Andes Mountains, Amazon river, mouth of the Amazon. Invite the students to think about their distribution, to speculate about processes that could have created them. In this phase, there are no wrong answers.

You can lead the students to observe the following features:

- Color differences: Greener area in the middle of the picture that corresponds to the Amazon basin and Amazon jungle. Brownish areas show less vegetation. The blue in the oceans and white-blue around the eastern margin of South America mark the transition between continental and ocean crust.
- The subduction zone along the west part of the continent and its alignment with the location of the Andes Cordillera.
- The big river that transverses the continent (The Amazon River, born in the Andes, and covering the whole width of the continent). Make them think about the function of the rivers (transporting water, sediment and nutrients).
- Ocean floor has long, nearly E-W features (These are fracture zones, divergent tectonic boundaries).

***Moniya Aména* Narrative**

(Suggested Activity # 2)

Invite an Elder or cultural authority to class to narrate the *Moniya Aména* myth to explain the origin of the Amazon. Alternatively, instead of having the Elder come to class, the students can go visit his *maloca*[5]. If none of these options are available, the teacher may obtain permission to tell the story to the students. Be sure to explain that this is a deep, cross-cutting myth, that can refer to several things (food, agriculture, plant taxonomy, etc.), but that will be explored in the context of the origin of the big rivers and the Amazon rainforest.

Weathering, Erosion and Deposition

(Suggested Activity # 3)

This activity helps students understand the concepts of weathering, deposition and erosion. This lesson is modified from Watts & Heaton (2014).

1- Break the students into three groups. One group is "Weathering", another group is "Erosion" and the third group is "Deposition". Make room in the classrooms so students can line up in front of each other and run between them.

2- Make cards with the name of the team, add tape to the back and stick the note on the forehead of the children (without covering their vision field).

[5] Traditional dwelling, where knowledge is transmitted.

3- The “weathering” team gets a sheet of paper that represents the Andean rock they will break down.

4- At the start of the activity the "weathering" students will start ripping tiny pieces of their "rock" and handing it to the "erosion" students. The "erosion" students will be running their tiny piece of "rock" to the "deposition" students at the back of the classroom to weathering. Those students will start making a delta with the tiny pieces on their assigned desk. Their job is to cover the entire desk with the tiny pieces they get from the erosion people. Note: “Weathering” students can only rip the paper into tiny pieces and hand it to the “Erosion” student one piece at a time (no ripping it all over the floor!).

5- During the activity ask questions to make sure students are understanding the concepts, or to identify misunderstandings that could arise.

6- Exit card: Assess the understanding of the big picture by asking students to write in a piece of paper:

   a. What is the big point that you learned in this class today?

   b. What would you like to learn more about?

The History of Amazonia

(Suggested activity # 4)

1. Before starting ask students to remember the *Moniya Aména* tree story. Highlight the points that are similar to the Western science model for the evolution of the Amazon: Absence of big river first (it was a tree); extensive flooding (lake at the tree's base); formation of river (when the tree was felled).
2. Narrate the evolution of the Amazon according to Western science. Use a story format to introduce the Western science knowledge about Amazonia formation. (Avoid names of geologic time, speak in ages instead).
3. Ask students to draw a cartoon, or a sequence of events that shaped the Amazon according to the Western science narrative and in the oral story. You could also split the classroom in two, and ask one side to illustrate (poster or presentation) or dramatize the traditional story, and the other part of the class will do the same with the Western story. Then the students will present their work in front of the class.
4. Ask students to identify common points and differences between the *Moniya Aména* and the Western Science story. Ask students if they think that a comparison between the two is useful, possible and appropriate. Make sure to show where the comparison is not applicable or breaks down.
5. Finally, the attention of students is directed to the part of the indigenous story that shows how different animals evolved from parts of the tree. Make the comparison to the co-evolution of landscape and species.

6. Exit card: In a piece of paper, students should summarize the main events that shaped the Amazon. Main events include: flooding due to an expansive lake, creation of the river, and the origin of the rain forest and evolution of species.

Integrating Acquired Knowledge

(Final activity)

Now that students know the key concepts of weathering, erosion, and deposition; that they understand the connection between the features in the continent, and that they've learned about the formation of the Amazon rainforest, ask students to explain:

1. The spatial distribution of the main features observed in the google image (Andes Range, Amazon Basin, Amazon River, delta of the Amazon River)
2. How have the weathering, erosion and deposition acted in the Andes–Amazon region through time.
3. What is the relation between the uplift of the Andes and the origin of the Amazon River?
4. Invent a story in which the Andes were not uplifted. What would happen to the Amazon rainforest?
5. In the *Moniya Aména* story, create an alternative ending for the story, in this new version, the tree could not be felled.

UNIT 2: EARTH SYSTEMS. –WHAT PROCESSES AFFECT OUR PLANET?

Subject Area: Natural Sciences

Length of unit: Four classes

Brief description of unit

The unit is designed for 5th grade students, it introduces Earth systems and system thinking. The unit makes the local knowledge relevant by introducing the three-world model of the Uitoto. It engages the sense of place through a short fieldtrip to a nearby location in which students can apply the concepts to observations of the natural world.

<table>
<tr><th colspan="2">STAGE 1 - DESIRED RESULTS</th></tr>
<tr><td rowspan="6">Established Goals. Standards

This lesson meets the NGSS 5-ESS2A.

• Students will examine the parts and processes of simple and complex systems.
• Students will apply new language for describing the various parts of systems.
• Students will practice using a framework for thinking about the Earth as a system.
• Students will practice identifying an Earth system in the local environment.</td><td>Enduring Understanding</td></tr>
<tr><td>Earth is a complex system of interacting rock, water, air, and life (Earth science big idea #3; ESLI, 2010)</td></tr>
<tr><td>Essential Questions</td></tr>
<tr><td>• What makes a system?
• What are the parts of the system?
• What are the processes within the system?
• What are the inputs and outputs of the system?
• Where does the energy come from to enable the system to function?
• What does the whole system do?</td></tr>
<tr><td>Essential Ideas</td></tr>
<tr><td>ESS2.A: Earth materials and systems. Earth’s major systems are the geosphere (rock), the hydrosphere (water), the atmosphere (air), and the biosphere (living things, including humans). These systems interact in multiple ways to affect Earth’s surface materials and processes. The ocean supports a variety of ecosystems and organisms, shapes landforms, and influences climate. Winds and clouds in the atmosphere interact with the land (NGSS, 2013.)

Uitoto Indigenous knowledge. At the highest level, the earth can be divided in: the world above (kofobiko), the world below (anabiko), and this world (biko). The world above corresponds to the sky, or atmosphere, the world below corresponds to the rocks, or geosphere, and this world corresponds to the living things and water or biosphere and hydrosphere. The different worlds interact between each other.</td></tr>
</table>

STAGE 2 – EVIDENCE

Assessment Evidence

Performance Expectations.

5-ESS2-1. Develop a model using an example to describe ways the geosphere, biosphere, hydrosphere, and/or atmosphere interact. Examples could include the influence of climate on the water level of rivers, and wetlands; the influence of the Caquetá river on the landscape (canyon and terraces), and the influence of the Andes range in continental precipitation patterns. (The geosphere, hydrosphere, atmosphere, and biosphere are each a system.) [Assessment Boundary: Assessment is limited to the interactions of two systems at a time.] (NGSS, 2013.)

Other assessments.

Test: included in the guide: Thinking about systems (see suggested activities).

"I Learned" statements: Before class ends, with perhaps three minutes remaining, the teacher asks students to write "I learned..." on a sheet of paper and then complete the sentence (Bond & Evans, 2011). This is a metacognitive assessment.

STAGE 3 – LEARNING PLAN

Learning Activities

a. Activate prior knowledge: Ask your students to describe something that is a system. What makes it a system?
b. State the goals of the unit.
c. Teach the class explaining the students what a system is,
d. Ask students to complete the guide "Thinking about systems" (AGI, 2013)
e. Ask students to develop an Earth systems model (performance actitvity). Make sure to tell your students specifically what you are looking for, and how they will be graded (Example, if you choose to have your students to a concept sketch explain the parts it should have (labels, connectors, explanations), also include the rubric for the assignment). The model development activity could start in class and be finished at home.
f. Extension. –Field trip. In this activity, a short excursion nearby helps students to apply the knowledge about Earth systems in their local environment. A field trip guide is attached as a suggested activity, it was modified from An introduction to Earth systems; AGI http://www.agiweb.org/education/aapg/invest/invest12.html).

References

American Geoscience Institute AGI. (2016). Thinking about systems. *Visiting Geoscientists. An outreach guide for geoscience professionals*. Retrieved from http://www.agiweb.org/education/aapg/invest/invest11.html

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Thinking About Systems

(Suggested activity 5.1; worksheet for students)

Goals

1. Examine the parts and processes of simple and complex systems.
2. Apply new language for describing the various parts of systems.
3. Practice using a framework for thinking about the Earth as a system.

Your Ideas

When you made observations of the natural world, you were looking at parts of the Earth system. You will learn to connect these and other parts. As you develop these connections, you will gain a better understanding of how the Earth works. The more you practice systems thinking, the clearer the picture of the Earth as a system will become.

Your scientific study of the Earth system will involve posing questions and seeking answers about how the Earth works. In this investigation you will learn that thinking about things as a system is very useful when asking scientific questions and developing ways to answer them.

- Describe something that is a system.
- Describe something that is not a system.
- Why is one a system but the other is not?

Record your ideas in your notebook. Share your thinking with others in your group and with your class.

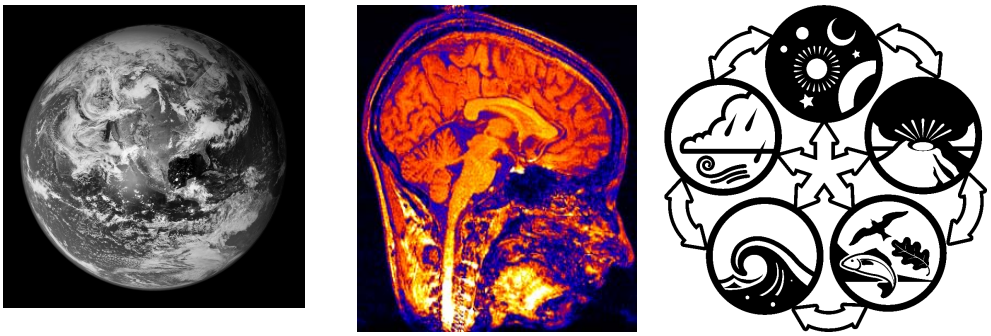


Figure C.2 The way that Earth works is very complicated, but thinking of it as a system enables us to break it down into smaller pieces that are easier to explore and understand.

Your Experience

Materials

- Stapler
- Poster paper
- Colored pens and pencils

Part A: Thinking about systems

The Earth is made up of an almost endless number of parts. Mountains, volcanoes, rivers, soil, oceans, clouds, rain, trees, and insects are only a few examples. The Earth's many parts work together to cause winds, tides, volcanic eruptions, earthquakes, ocean currents, evolution of life, and much more. How can you simplify and organize this large and complex place so it is easier to understand? One way is to think of the Earth as a system.

A system is a group of related features or objects that are organized in some way. Different parts of a system interact with each other. This causes a system to function (do something) in some way as a whole. Every system has a driving force that makes it work.

To understand systems, you are going to examine a stapler as a model of a system. You will explore the question "how is the stapler a system?"

Figure C.3 A stapler is simple machine but still a system.

1. With your group, develop a method for exploring how the stapler works as a system. Describe your method as a series of steps. Present your group's ideas to the class. Don't worry if you feel that you don't know much about systems. Your task is to begin thinking more about them, and start to develop some skills in systems thinking.
2. Apply your method to the stapler and show your findings on a poster.
3. Discuss your ideas about systems thinking with other groups. Be open about your ideas and thoughts. Talk about different types of systems and about some of the ways of thinking about them. Doing this will help you to see new ways of looking at complex things as systems. You may also begin to develop new words for describing things that are common to most systems.

Systems Thinking

Earth system scientists are very curious about how one part of the planet affects another. To understand the connections among parts of the Earth, they view the natural world as a system.

There are many steps to thinking about systems. First, you identify the **parts** of the system. The parts are the elements (or things) within the system. Then, you look at how the various parts are connected to each other. When parts of a system interact, a **process** of some kind operates. A processes causes change in a system. Finally, you think about the **function** of the system and how the parts work together as a whole to do work of some sort.

Think back to the stapler. New staples and springs are two of the many parts of the stapler system. Because these parts are connected, they affect one another. Various processes act to change a new staple into a used one. Pushing down on the end of the stapler causes the staple to change shape by being pressed between different parts. The function of the stapler as a whole is to attach pieces of paper together.

In any system, there can be a large number of parts and processes. Both are very important features of systems, and without either of them, nothing would happen. A system relies on all of its parts to function and will not function properly if some parts are removed.

Matter and **energy** are two important parts of all systems. Energy provides a system with the capacity to do work. Matter is the material in a system, literally the stuff the system is made of. Certain processes add energy and matter to a system. These are known as **inputs**. After an input enters into a system, a process may act on it and cause it

to change in some way. Also, newly added matter can be combined with matter already in the system, such as the input of fresh water from a river flowing into the ocean. Some processes, called **outputs,** remove matter and energy from a system. Think about the loss of water from the surface of the ocean because of evaporation.

Remember that looking at systems is all about making connections between different things. Often the output from one system can be the input into another system. For example, water that evaporates from the oceans becomes part the atmosphere as water droplets in clouds. The movement, or flow, of matter and energy enables systems to keep working.

Part B: Using Systems Language

Now that you have learned about the various aspects of systems, return to your poster of the stapler and apply your new knowledge.

1. Label the parts of the stapler system.

2. Describe the processes that operate while the stapler system works.

3. Identify the following in the stapler system:

a) inputs (enter)

b) a flow of matter

c) a flow of energy

d) something that changed

e) the process that caused the change

f) outputs (leave)

4. Can the stapler system work without energy being added to it? Why or why not?

Compare your first description of how the stapler works with this new one that uses systems language. Describe two ways that systems thinking helped you think about how a stapler works.

Ways of Systems Thinking

You used a specific way of thinking to look at the stapler as a system; this process is called **systems thinking**. Systems thinking involves asking five basic questions:

- What are the parts of the system?
- What processes cause change in the system?
- What are the inputs and outputs of the system?
- Where does the energy come from to enable the system to function?
- What does the whole system do?

Systems Thinking and Scientific Inquiry

Earlier you began looking at science as a process for understanding the natural world. Now that you have practiced thinking about systems you will be able to organize your thoughts and explain things more clearly as you study the Earth and its systems.

Part C: Applying systems-thinking

1- Identify a human-made system and a natural system. Use the list in Table C.1 to help you.

Table C.1

school	rivers	computer
human body	pond	soccer team
bailes (traditional ceremonies)	ant hill	*maloca* (traditional dwelling)
chagra	cell phone network	flashlight

Apply systems thinking to describe and explain each system. Use diagrams to show how the parts are organized, and how energy and matter flows through each one

Part D: The Earth System

Scientists describe the Earth system as containing five major systems, called spheres. They are not called spheres because of shape, but because of the parts and processes they contain. Each sphere is different from the other spheres. Through the interaction of these spheres, the planet functions as an even larger system.

1. Read about each sphere as it is described in "The Earth System" reading passage on the next page.

a. Which of these spheres were you already familiar with?

b. Which of these spheres are you learning about for the first time?

2. You learned that systems thinking helps you to organize your ideas. Read through the observations you recorded in Investigation 1.1 and think about which of the Earth's major systems they are part of.

    a. For each observation, decide which sphere it belongs to.

    b. State whether the observation is a part or a process in that sphere.

    c. Identify and label any inputs and outputs.

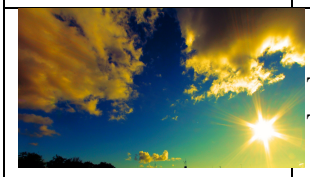
    d. Record any ideas you have about how energy enables any of the processes you described.

Provide an example of one sphere interacting with another.

The Earth System

Table C.2.

	The **geosphere** includes the crust and the interior of the planet. It contains all of the rocky parts of the planet, the processes that cause them to form, and the processes that have caused them to change during Earth's history. There are thousands of parts and processes in the geosphere. It has parts that can be as small as a mineral grain or as large as the ocean floor. Some processes act slowly, like the gradual wearing away of cliffs by the sea. Others are more dramatic, like the violent release of gases and magma during a volcanic eruption.
 	The **fluid spheres** are the liquid and gas parts of the Earth system. The **atmosphere** includes the mixture of gases that surrounds the Earth. The **hydrosphere** includes the planet's water system. Its parts include oceans, lakes, rivers, and frozen water in glaciers. A special property of the fluid spheres is that their materials flow. Processes in the fluid spheres include the water cycle, the circulation of the atmosphere and oceans, and weather.
	The **biosphere** contains the living and once-living parts of the Earth system. It is organized into complex webs of microorganisms, animals, and plants. It also includes dead and decomposing living things and special molecules from once-living material. Processes vary from simple predator — prey relationships to changes over millions of years in the kinds of living things that make up communities. This part of the Earth system is distributed widely across the Earth, from the cold dark depths of the oceans, to the thick rainforest near the Equator.

	The **exosphere** includes the outermost part of the Earth system and beyond. This system connects our planet with other bodies in the Solar System. Our nearest neighbor, the cratered Moon, is part of the exosphere, as are the other planets that orbit our star, the Sun. Despite the great distances of space, the Sun, the Earth, and its neighbors are connected in various ways that affect processes at work on Earth today. It does not end there. Our Solar System is contained within a vast region of space and stars called the Milky Way galaxy.
Uitoto Traditional knowledge	
	For the Uitoto, the geosphere would be equivalent to the ***anabiko*** (or world below)
 	This world, or ***biko*** is where the biosphere and hydrosphere can be found. They interact among them and with the other worlds.
	The world above, or ***kofobiko***, is where the atmosphere is. The world above interacts with ***anabiko*** and ***biko***.

Your Understanding (Test; assessment)

Parts

1. What are the parts of a system?

2. Name a system and identify two parts in that system.

Processes

3. For each pair of items below, identify which item is a part of a system and which is a process within a system.

a. stomach; digestion

b. flooding; river

c. canyon; water erosion

d. orbit; Earth

4. What do processes do? Describe a process in the natural world.

5. Think about a canoe, or a boat, as a system (it can be a motorized boat). Using a simple sketch with labels, answer the following questions.

a. Name one part of the system.

b. How is that part of the system itself made of parts?

c. What are **all** of the parts of the system?

d. How does each part relate to the others (the processes)?

e. What does the system do as a whole?

6. How do inputs differ from outputs?

7. Identify one part of a stapler. If this part were removed, how would this affect how the system works?

8. What does it mean when we say that a stapler system is more than the sum of its parts?

9. What are the key questions to systems thinking?

10. If someone gave you a new system and asked you to describe it, what would you do first? Second?

11. How did using a systems approach make thinking about systems more difficult? Easier?

12. Look back at your responses to the Your Ideas questions. How have your ideas about systems changed as a result of this investigation?

Short Field trip.

(Suggested activity 5.2)

Preparation.

1. This investigation is set up for groups of four. Look for help of another teacher or responsible adult if your group is large when you take the students outside to make observations.
2. Make a copy of the 'Earth Systems Concept Map Poster' (http://www.agiweb.org/education/aapg/invest/EarthSystemsConceptMap.pdf)
3. Collect all the materials in the list below and organize them into one Tool Bag for each group of four students.
4. Make photocopies of the handouts (Earth systems diagram and Earth systems concept map, example).
5. Run through the investigation yourself and record the data, just to see how long it takes. Adjust the timing to the class period, remembering that you will need time to introduce the investigation, clean up afterwards and re-set up for the next class (if you are working with more than one class)

Lesson time. 55-60 minutes.

Objective. Students will be able to make connections between the different spheres of the Earth system by observing their outdoors and categorizing their observations.

Materials. Provide students, in groups of four, with the following:

- Large sheets of poster paper

- Markers
- Earthy systems diagram (http://www.agiweb.org/education/aapg/invest/EarthSystemsDiagram.pdf)

All students:

- Notepads to record observations
- Pen or pencil

Purpose. This investigation is designed to help participants make connections between the different components of the Earth System (biosphere, hydrosphere, atmosphere and geosphere) through observing their local outdoor area, categorizing the observations into one or more "spheres", and drawing links between the spheres.

Investigation Question. What are the spheres of the Earth System and what parts and processes occur in these spheres?

Procedure.

1. (5 minutes) Hand out copies of the Earth Systems diagram to each group of three or four participants. Read the diagram over with the participants to make sure that they understand what each system comprises.
2. (10-15 minutes) Provide participants with paper. Take the students to a place outside the school where they can observe two or more components of the Earth system (e.g., the sky, the land, a water body, vegetation, etc.). They will write down as many observations about the outside world as they can. Encourage them to use not just their sight, but also their hearing, and senses of touch and smell as they make their observations. For example, they might not see any birds or insects,

but they might hear bird song and insects buzzing. They also might hear the wind or smell the forest. They can feel the dampness from dew on the grass or hear water flowing. (Make sure that students stay on task.)

3. (5-10 minutes) When they come back inside (or if you can do this outdoors, do it), ask them to work in small groups to categorize all their observations into the sphere where they best fit. Caution them that there will be overlap, but not to be concerned about that.
4. (10-15 minutes) Next, ask participants to work in their groups to make a poster showing the interaction of the parts and processes of the Earth's systems, using their observations as specific examples. They could use a concept map format for this, but they should feel free to use their creativity. They might want to use arrows between the different systems, for example, possibly using different colored arrows for positive and negative effects. They could also use Post-it notes to show processes or examples.
5. (10-15 minutes) When each group finishes its poster, they should have the opportunity to present what they have discovered about the parts and processes of the Earth systems and the links between them. Help them think about the bigger picture of the local place within the Amazon basin, then within the Andes-Amazon, and then, within the globe, applying systems thinking.
6. (2 minutes) Thank students for their time and attention.

UNIT 3: DISTRIBUTION OF WATER IN THE PLANET

Subject Area: Natural Sciences

Length of unit: Four classes

Brief description of unit: The unit is designed for 5th grade students. It explains the distribution of water on Earth using a demonstration by the teacher. It emphasizes the importance of fresh water for life in the planet. The lesson addresses the local environment because it includes the percentage of water transported by the Amazon River.

<table>
<tr><th colspan="2">STAGE 1 - DESIRED RESULTS</th></tr>
<tr><td rowspan="6">Established Goals.
Standards.

This unit addresses the Colombian competency for 5th grade: Justify the importance of water to sustain life, and the NGSS 5-ESS2B

• Students will identify the percentage of freshwater on earth that is available for human use.
• Students will describe the distribution of different types of water in the globe.
• Students will explain why it is important to preserve the water we currently have available for biotic use. (Goals were taken from water, water everywhere activity)</td><td>Enduring Understanding</td></tr>
<tr><td>Earth is a water planet (Earth science big idea #5; ESLI 2010).
Water is essential for life.
Fresh water is key for the Amazon rainforest.</td></tr>
<tr><td>Essential Questions</td></tr>
<tr><td>• Where does water occur on our planet?
• What fraction of the water of the planet is available for human, and other living beings, use?
• What fraction of the fresh water is transported by the Amazon River?</td></tr>
<tr><td>Essential Ideas</td></tr>
<tr><td>ESS2.C: The Roles of Water in Earth’s Surface Processes.
Nearly all of Earth’s available water is in the ocean. Most fresh water is in glaciers or underground; only a tiny fraction is in streams, lakes, wetlands, and the atmosphere (NGSS, 2013).

The Amazon River accounts for 1/5 of the earth’s fresh water, it is the river that carries the largest volume of water in the world.</td></tr>
</table>

STAGE 2 – EVIDENCE

Assessment Evidence

Performance expectations

5-ESS2-2. Describe and graph the amounts and percentages of water and fresh water in various reservoirs to provide evidence about the distribution of water on Earth. [Assessment Boundary: Assessment is limited to oceans, lakes, rivers, glaciers, ground water, and polar ice caps, and does not include the atmosphere.]

Other assessments

Test included in the demonstration: Water, Water Everywhere

Describe and graph the fraction or percentage of fresh water transported by the Amazon River.

STAGE 3 – LEARNING PLAN

Learning Activities

1. Activate prior knowledge: Begin by asking students to write where do they think most of the water of the planet is, give the options: Rivers, oceans, ground water, ice caps and glaciers, swamps or lakes.
2. Perform the suggested demonstration and activities 'Water, Water Everywhere' (Taken from: Population education. org), pose the discussion questions in class, and assess learning.

References

Earth Science Literacy Initiative. (2010). *Earth science literacy principles: The big ideas and supporting concepts of Earth science*. Arlington, VA: National Science Foundation. Retrieved from: http://www. earthscienceliteracy. org/es_literacy_6may10_. pdf.

NGSS Lead States. (2013). *Next Generation Science Standards: For States, By States (Earth Science Systems)*. Washington, DC: The National Academies Press.

Population Education. (2015). *Water, Water Everywhere. Student activity.* Retrieved from https://www.populationeducation.org/sites/default/files/water_water_everywhere.pdf

Water, Water Everywhere

(Suggested activity #1)

Method

Students observe a demonstration of how much water is available on the planet for human consumption.

Materials

- Large, clear container (can hold at least one gallon)
- Medium clear container (can hold at least one cup)
- 3 small clear containers (test tubes, juice glasses, etc.)
- Dropper
- Water
- Soil
- Blue food coloring
- 1 cup measure
- Full set of measuring spoons
- Masking tape

Part 1: Water Demonstration Procedure

1. Explain to students that freshwater is a term used for the water we use in our daily lives – for drinking, washing, and growing food. It is found in rivers, streams, many lakes, and underground. Freshwater comes out of faucets and hoses. Ask the students,

"Can you think of any other types of water? Water that we cannot drink or use to grow plants?" (Saltwater), and, "Where do you think most of the water is on Earth?" (Oceans).

You may choose to show them the picture in Figure C.4.

Figure C.4. An image of the 'blue marble' could lead students to think that most of the water found on earth is in the oceans. Image modified from NASA, astronomy picture of the day, retrieved from: http://antwrp.gsfc.nasa.gov/apod/image/0304/bluemarble2k_big.jpg

2. Set out all the containers, measuring cups, and spoons where the class will be able to see them. Add about a half inch of soil to the second small container.

3. With the masking tape and marker, make a label for each of the four water categories you'll be discussing: (1) Salt water – 97% (2) Frozen – 2% (3) Underground – 0.7% (4) Surface water – 0.3%. Leave the labels stuck to the edge of the counter at this point; you'll attach them to the containers later.

4. Fill the large clear container with 6 cups of water and add a few drops of blue food coloring.

5. Ask, “How much of the earth’s surface is covered by water?” (70 %). You may want to pull out a map of the world or globe again so the students can make better estimates (e.g., Figure C.4). Point out the full container. “This represents all of the water on the planet.”

6. Scoop three tablespoons from the big container into the medium container. Attach the “Salt water” label to the large container and hold it up again. “This represents the 97 % of the earth’s water that is salty. Most of it is found in the oceans.” Hold up the medium container containing the 3 tablespoons of water. “This represents the other 3 % of the world’s water – freshwater.” You’ll now divide the freshwater to find what amount is accessible to humans.

7. From the medium container, measure out two tablespoons and pour into the first small container. Attach the “Frozen” label and hold it up. “This amount represents the 2 % of the earth’s water that is frozen in glaciers and ice caps.”

8. Again from the medium container, measure out 2 teaspoons and pour into the second small container with soil. Attach the “Underground” label and hold it up. “This amount represents the 0.7 % of the earth’s water that is located under the ground.”

9. Pour out the remaining water (1 teaspoon) from the medium container into the third small container. Attach the “Surface water” label and hold up. “This amount

represents the 0.3 % of the earth's water that is on the surface of the planet – in places such as rivers, lakes, and streams."

10. Ask the students, how much of the Surface Water (third small container) flows through the Amazon River? Take the glass dropper and ask them, how much should I withdraw from the teaspoon to represent the river? If the teaspoon is 5 mL, and the dropper is 2 mL, then half a dropper of water represents how much water is carried by the Amazon River. Hold up the dropper "This represents 1/5 of the fresh water on the continents. The Mississippi River, which is one of the longest river of North America, transports half this volume."

Discussion Questions

1. Which of these kinds of water could we use for daily purposes such as drinking, washing our bodies and clothes, cooking and making *cagüana*?

   The 0.3 percent of surface water and maybe some of the water underground. [Holdup containers #3 and #4.]
2. What sources of water can't we use for those purposes?

   Salt water, water that is frozen, and some of the water underground if it's very deep. [Hold up containers #1, #2, and #3.] Even if water is fresh, it can't be used if we can't access it.
3. Do you think it's important for us to be careful with this fresh, accessible water? Why? What are some ways we can be careful to preserve this water?

   Yes. We need water for our survival but can only use a small portion of the water on earth. We can be careful with water by conserving it through actions such as

turning off the faucet when brushing our teeth, watering our plants in the evening, not littering the river, maintaining the river channel clean, etc.

4. As our population grows, does the demand for clean, freshwater increase or decrease? *(Increase – more people will need water.)*

   As more people use the limited amount of water on earth, what will happen to our reserves, will they increase or decrease? *(Decrease – more people are using water.)*

5. In addition to more people using more freshwater, what else might decrease the amount of freshwater available?

   If water becomes polluted, it can no longer be used. Water can become polluted from many things including the following: mining, fertilizer run-off from farms, waste being poured down storm drains, acid rain, oil spills, etc.

6. Consider that the teaspoon represents the water of ALL the rivers, streams, lakes on earth. The Amazon River carries 20 % of ALL the fresh water in the world. That makes it one of the most important rivers in the world. What would happen if its waters became polluted?

Learning Assessment

Have students write an exit slip to the following prompts:

a. It's important to take care of the freshwater we use because… (name two reasons)

b. To protect the earth's freshwater I will… (name two actions)

c. The thing that surprised me most today was…

d. I would still like to learn more about…